

EFFECTS OF NITROAROMATIC COMPOUNDS ON SPECTRA OF D-AMINO ACID OXIDASE

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SUMMARY D-amino acid oxidase (D-amino acid oxygen oxidoreductase E.C. 1.4.3.3.) was found to bind various nitroaromatics with characteristic spectral changes similar in form to those caused by benzoate and its analogues. Nitrobenzene and o-nitroaniline were found to be competitive inhibitors of the enzyme. Dissociation constants of nitroaromatics tested were larger than those of the carboxylates except in the case of o-nitroaniline. Dinitrophenol was also found to bind to the enzyme with almost the same value of dissociation constant as nitrobenzene; on the other hand, dicarboxylates had no complex ability (3).

INTRODUCTION

Benzoate and its derivatives are well known potent competitive inhibitors of D-amino acid oxidase and binding of benzoate to the enzyme causes pronounced spectral changes (1-4). The characteristics of binding of inhibitors to the enzyme are important for understanding the relationship between structure and function. The essential characteristic for binding of inhibitors to the enzyme has been found not to be the aromatic ring but a carboxyl group (3). The participation of some basic group of the enzyme in the binding of inhibitors and substrates has been implicated from this characteristic. The involvement of several amino acid residues in the active site were proposed for the binding of substrate to the enzyme from kinetic experiments (4) and chemical modification studies (5-16).

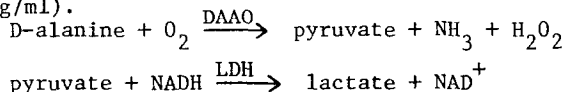
This paper reports further characteristic spectral changes by the binding of nitroaromatics, competitive inhibitors, to D-amino acid oxidase.

MATERIALS AND METHODS

Materials D-amino acid oxidase was purified from hog kidneys by the procedure of Curti et al (17). Benzoate was removed by the method of repeated ammonium sulfate precipitation after addition of D-alanine (18). 1-Fluoro-2,4-dinitrobenzene (FDNB) was obtained from Daiichi Pure Chemical Co. Nitrobenzene, o-,m-,p-nitroanilines, o-,m-,p-nitrophenols and sodium benzoate were obtained from Wako Pure Chemical Co. NADH, FAD and D-alanine from Sigma and crystalline rabbit muscle lactate dehydrogenase from Boehringer Mannheim. All reagents were of analytical grade.

Spectrophotometric analysis Absorption spectra were recorded with an Aminco DW-2a TM UV-VIS spectrophotometer in the split beam mode, in 20 mM pyrophosphate buffer pH 8.2, 19-20°C. The nitroaromatic compound, dissolved in 5% ethanol was added to the enzyme and reference cuvettes in equal volumes, and spectra were determined. The final concentration of ethanol was adjusted to less than 1%; this concentration proved to have no significant effect on the spectra or the catalytic activity of the enzyme. To obtain difference spectra between a nitroaromatic-enzyme complex and free enzyme an equal volume of the enzyme solution was added to each of two quartz cells. After the base line absorption was recorded, 10-30 μ l of the nitro compound in 5% ethanol solution was added to the sample cell and an equal volume of 5% ethanol solution was added to the reference cell.

Kinetic analysis Kinetic analyses were performed at 25°C in a reaction mixture containing 20 mM pyrophosphate buffer pH 8.2, 10^{-5} M FAD, 4×10^{-3} M NADH, various concentration of D-alanine and nitroaromatics. The enzyme activity was determined by the decrease in A_{340} of NADH in the following coupled assay system (19) with excess rabbit muscle lactate dehydrogenase. (0.17mg/ml).



Nitroaromatic compounds dissolved in 5% ethanol were added to reaction mixtures. The ethanol concentrations of all the reaction mixtures were less than 1%. This concentration of ethanol has no significant effects on either DAAO or LDH activity. Nitroaromatic compounds also have no significant effects on the catalytic activity of LDH under the experimental conditions. D-amino acid oxidase was preincubated for 10 minutes at 25°C in 20 mM pyrophosphate buffer pH 8.2 containing 3×10^{-5} M FAD and 5×10^{-2} M D-alanine before diluting 300-fold into the assay mixture as previously described (16).

RESULTSEffects of nitrobenzene on the spectrum of D-amino acid oxidase

Figure 1 shows the spectral shift of D-amino acid oxidase with nitrobenzene. Figure 2 shows the difference spectra obtained in a titration experiment. For the sake of clarity, several intermediate spectra have been omitted. The decrease in absorbancy in the region of 320-390 nm and 400-470 nm, as well as the increase in the region of 480-530 nm are very similar to those with benzoate, although the affinity of the compound to the enzyme and

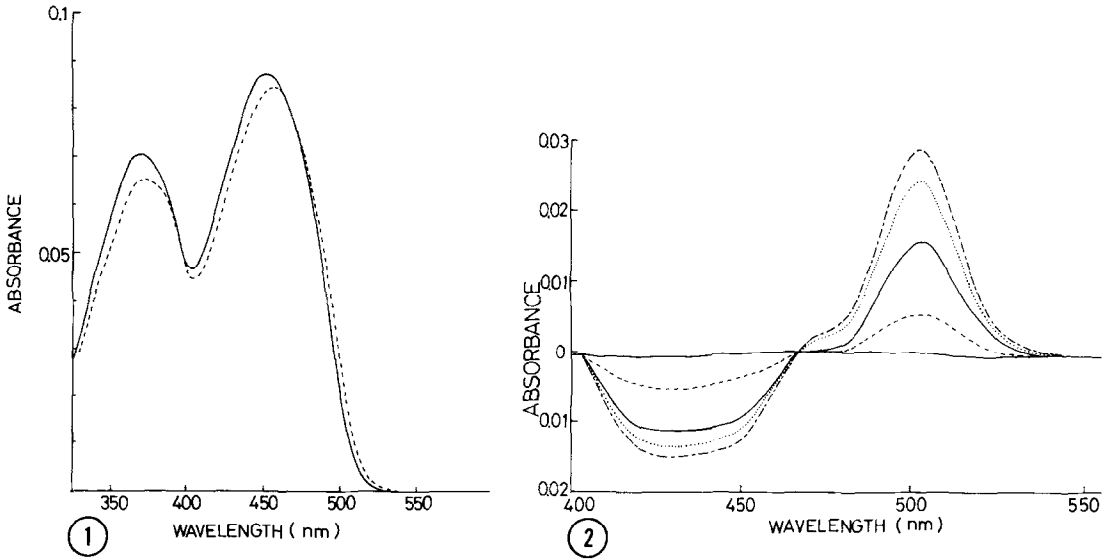


Figure 1. Effect of nitrobenzene on the spectrum of D-amino acid oxidase. Enzyme ($7.7 \times 10^{-6}M$) in 20 mM pyrophosphate buffer pH 8.2; —, after addition of nitrobenzene to a final concentration of $4 \times 10^{-4}M$; - - - - -.

Figure 2. Difference spectra between enzyme-nitrobenzene complex and free enzyme. The enzyme concentration was $2.4 \times 10^{-5}M$ in the same buffer as in Fig. 1. Curve - - - - -; at a nitrobenzene concentration of $1.06 \times 10^{-4}M$, —; at $2.4 \times 10^{-4}M$,; at $4.9 \times 10^{-4}M$, - - - - -; at $7.4 \times 10^{-4}M$.

its solubility in aqueous solution are so low that saturation could not be achieved. The fact that nitrobenzene was bound to the enzyme and produced the spectral effects suggests that the nitro group may mimic the carboxyl group in this binding. If, so, other nitroaromatics, such as nitroaniline or nitrophenol, should produce similar effects to those of aminobenzoate or hydroxybenzoate.

Effects of other nitroaromatics

Amino substituted benzoates, such as o-, m-, p-aminobenzoate, produce some degree of charge transfer-like long wavelength absorption (3). Amino substituted nitrobenzenes also produced very similar effects. Figure 3 shows the spectral shift obtained on titration of the enzyme with o-nitroaniline. Intermediate spectra have been omitted. The spectra show a decrease in absorbancy in the region of shorter wavelength below 500 nm, but a marked increase in the region of 500-640 nm, typical of a charge transfer transition.

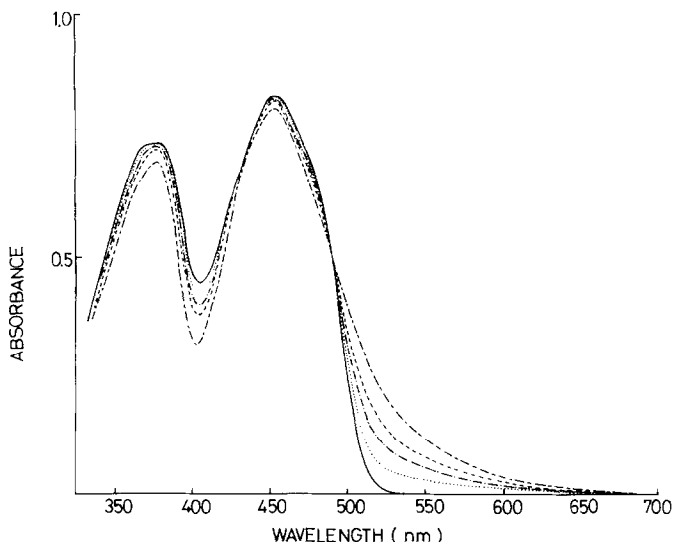


Figure 3. Effect of *o*-nitroaniline on the spectrum of D-amino acid oxidase. Curve —; enzyme ($7.4 \times 10^{-5} M$) in the same buffer as in Fig.1,; after addition of 0.27 moles nitroaniline per mole enzyme FAD, -.-.-; 0.55 moles, -----; 0.82 moles, - - - -; 2.1 moles.

It should be noted that the maximum wavelength is about 520–530 nm which is rather shorter than that with anthranilate (560 nm). These spectral changes are to a large extent complete by the addition of 1 molecule of *o*-nitroaniline per molecule enzyme bound FAD, suggesting relatively high affinity of this compound for the enzyme. This is illustrated further in Fig. 4, where the increase in absorbancy at 565 nm is plotted against the amount of *o*-nitroaniline added. The binding has a stoichiometry of 1 : 1 with respect to flavin. From the result of Fig. 4 the dissociation constant of *o*-nitroaniline is calculated to be $6.9 \times 10^{-6} M$. The other nitroanilines were also found to produce similar spectral effects, but with affinities much weaker than *o*-nitroaniline. *o*-nitrophenol shows a very small spectral shift and very weak long wavelength absorbance. *m*- or *p*-hydroxy substituted nitrobenzene also shows small spectral effects, but the affinity was found to be much weaker than *o*-hydroxy substituted nitrobenzene. (Spectra not shown.)

Effects of 1-Fluoro-2,4-dinitrobenzene

The results described suggest strongly that the nitro group is behaving like the carboxyl group in binding of compounds to the enzyme.

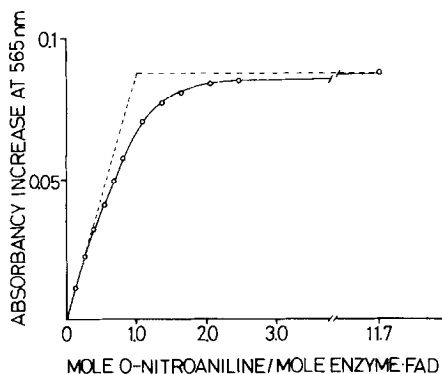


Figure 4. Increase in absorbancy at 565 nm on titration of D-amino acid oxidase with o-nitroaniline. Conditions as in Fig. 3. The concentration of enzyme bound FAD in this experiment was $7.4 \times 10^{-5} \text{M}$.

Although monocarboxylic acids complex with D-amino acid oxidase, the presence of additional carboxyl groups in analogous compounds such as phthalate, terephthalate, and fumarate prevent the formation of complexes (3). However, 1-Fluoro-2,4-dinitrobenzene clearly binds to the enzyme, as shown in Fig. 5. An initial rapid shift was seen, which was very similar to that with nitrobenzene, and was followed by the second slower shifts, which were parallel with the loss of catalytic activity as shown in inset. This activity loss of the second phase has been found to be due to modification of a tyrosyl residue in the active site of the enzyme (16). Another dinitro compound, dinitrophenol, was also found to bind to the enzyme.

Inhibitory kinetic properties of nitroaromatics

The kinetic properties of nitrobenzene and o-nitroaniline were investigated. Both were found to be competitive inhibitors of the enzyme. The K_i values were found to be $8 \times 10^{-4} \text{M}$ and $4 \times 10^{-6} \text{M}$ respectively. These values are in reasonable agreement with K_d values obtained by titration experiments. (cf. Table 1)

DISCUSSION

It is clear from the spectral effects which they produce that nitroaromatics are bound to D-amino acid oxidase. From the titration experiments, it can be seen that the affinity of nitro compounds is too low to achieve full

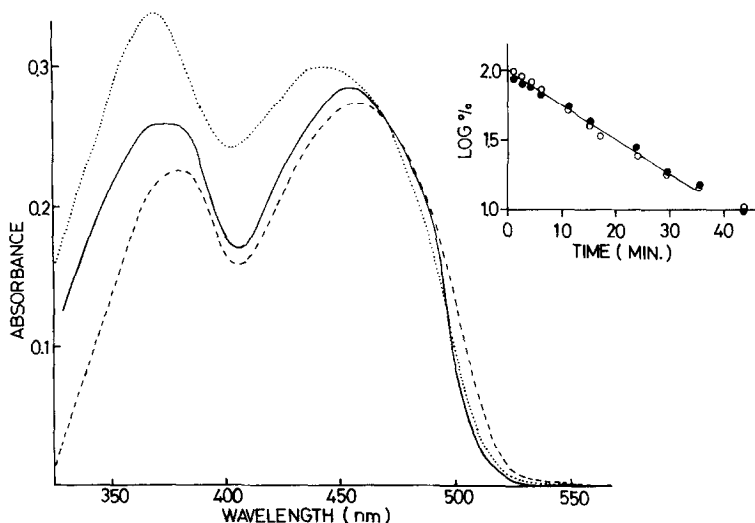


Figure 5. Effect of 1-Fluoro-2,4-dinitrobenzene on the spectrum and activity of D-amino acid oxidase. —; native enzyme of 2.5×10^{-5} M concentration, - - - -; immediately after addition of excess FDNB of 9×10^{-4} M concentration,; 44 minutes after addition of FDNB. Inset shows the time course of enzyme activity and absorbance decrease at 510 nm. (●; log percent activity, ○; log percent decrease of A_{510}). Enzyme assay; The reaction of the enzyme with FDNB was stopped at each time point by withdrawing aliquots and mixing with 0.05 M phosphate buffer pH 7.5 containing 5 mM sodium benzoate. [Benzoate protects completely FDNB inactivation. (16)] The activity of each quenched sample was assayed spectrophotometrically with D-phenylglycine as substrate by the method of Fonda and Anderson (5). In this procedure preincubation was omitted.

Table 1.
Comparison of Dissociation Constants of Nitroaromatic Compounds and Carboxylates to D-Amino Acid Oxidase

Nitroaromatic Compounds	Kd (M)	Carboxylates*	Kd (M)
Nitrobenzene	4.4×10^{-4}	Benzoate	3.0×10^{-6}
o-Nitroaniline	6.9×10^{-6}	o-Aminobenzoate	2.0×10^{-5}
m-Nitroaniline	1.8×10^{-3}	m-Aminobenzoate	3.2×10^{-5}
p-Nitroaniline	2.9×10^{-3}	p-Aminobenzoate	3.4×10^{-4}
o-Nitrophenol	6.7×10^{-4}	o-Hydroxybenzoate	5.0×10^{-6}
m-Nitrophenol	$\sim 10^{-1}$	m-Hydroxybenzoate	4.6×10^{-5}
p-Nitrophenol	$\sim 6.8 \times 10^{-2}$	p-Hydroxybenzoate	1.9×10^{-4}
2,4-Dinitrophenol	2.3×10^{-4}	Phthalate	not bound

The dissociation constant of the nitroaromatic compound to the enzyme is defined by the expression $K_d = \frac{[\text{free enzyme}] \cdot [\text{free nitroaromatic compound}]}{[\text{enzyme-nitroaromatic compound complex}]}$ in 20 mM pyrophosphate buffer, pH 8.2, 19–20°C. × V. Massey and H. Ganther (ref. 3)

saturation except in the case of o-nitroaniline. The latter compound was bound to the enzyme with relatively high affinity ($K_d = 6.9 \times 10^{-6} M$) and the stoichiometry was found to be 1:1 with respect to flavin. Although other nitroaromatics did not achieve full saturation, the K_d values of those complexes were estimated from double reciprocal plots of absorbance changes versus concentrations of nitro compounds by assuming stoichiometries were 1:1 with respect to flavin. The concentrations of the enzyme were much lower than those of the nitro compound, and the intercepts were used to get maximum changes of absorbance and K_d values (Table 1). As seen in the table almost all K_d values of nitroaromatics are larger than those of the carboxylates except in the case of o-nitroaniline. Presumably the weaker charge of the nitro group compared with the carboxyl group, which may interact with a positively charged group of the enzyme, may be the reason for the lower affinity of these compounds. The high affinity of o-amino substituted nitrobenzene may be explained by another negative charge of a tyrosine residue of the enzyme, which would be close to the o-amino residue of nitro compounds as postulated by Nishino et al (16). These steric positionings were proposed from the results of FDNB inactivation of the enzyme, that the fluorine atom would be close to the tyrosine residue of the enzyme. The difference between dinitroaromatics and dicarboxylates in enzyme binding ability is probably due to the weaker electronegativity of nitro groups than carboxyl groups, which may prevent complex formation by interacting with another negatively charged residue of the active site of the enzyme. The fact that nitroaromatics are bound to the enzyme suggests that the nitro group may mimic the carboxyl group in the binding site of the enzyme. Recent evidence suggests that a guanidino group at the active site of the enzyme is concerned in this binding (16).

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