REACTION OF REDUCED FLAVINS WITH TETRANITROMETHANE

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SUMMARY

Tetranitromethane (TNM) was found to oxidize $FMNH_2$ and the reduced form of D-amino acid oxidase. During the oxidation of $FMNH_2$, TNM decomposes into the nitroform ion and NO_2^-. The reaction with $FMNH_2$ was completed within the mixing time of the stopped-flow apparatus (2.5 msec), whereas the reaction with the reduced enzyme could be monitored by the apparatus. The spectral change of the latter reaction indicated that a 2-electron transfer process occurred simultaneously with a 1-electron transfer. The occurrence of the nitroform ion was also demonstrated in the catalytic oxidation of D-arginine in the presence of TNM.

Tetranitromethane (TNM) has been extensively used to nitrate tyrosyl residues and to oxidize sulfhydryl groups of proteins (1). It was also used to modify enzyme-substrate intermediate (2). In the present study, TNM was found to be an electron acceptor in the oxidation of $FMNH_2$ and reduced D-amino acid oxidase [EC 1.4.3.3]. These reactions are of interest in flavin chemistry, since attention has recently been focused on the acceptor specificity of reduced flavin groups when bound to enzyme proteins (3,4).

MATERIALS AND METHODS

FMN and TNM were commercial products. D-Amino acid oxidase was prepared according to the method of Yagi *et al.* (5). The concentration of the enzyme was expressed in terms of bound FAD (ε_{455} = 11,300 M^{-1} cm^{-1}). $FMNH_2$ was prepared by irradiating FMN with visible light in the presence of EDTA (1.0×10^{-3} M) under anaerobic conditions (6). The reduced enzyme was obtained by adding D-arginine to the oxidized enzyme under anaerobic conditions.

In the study of the stoichiometry of the reaction of $FMNH_2$ with TNM, FMN and nitroform ion formed were determined spectrophotometrically [ε_{450} = 12,200 M^{-1} cm^{-1} for FMN (7), ε_{350} = 14,400 M^{-1} cm^{-1} for nitroform ion (8)], and NO_2^- was determined by colorimetric method using Griss-Romijn reagent (9). Absorbance was measured in a Beckman DK-2A spectrophotometer or a Shimadzu QV-50 spectrophotometer. Stopped-flow experiments were performed using a Yanaco SPS-1 stopped-flow spectrophotometer with a 2-mm cuvette. The details of the apparatus were described elsewhere (10). Oxygen consumption was measured polarographically using a Beckman oxygen sensor. All measurements were performed in 0.017 M sodium pyrophosphate buffer (pH 8.3) at 15°C, unless otherwise specified. TNM was dissolved in ethanol, and was diluted to the required concentration with the buffer.

RESULTS AND DISCUSSION

Oxidation of Free $FMNH_2$ by TNM —— The pale yellow color of an anaerobic solution of $FMNH_2$ (3.3×10^{-5} M) turned immediately to yellow upon addition of TNM (5.3×10^{-5} M), indicating that $FMNH_2$ was oxidized to FMN. Equimolar amounts of nitroform ion and NO_2^- were produced in coupling to the oxidation of $FMNH_2$ to FMN (Table I). This stoichiometry was also found in the reaction when the molar ratio of TNM/$FMNH_2$ was less than 1. The reaction of $FMNH_2$ (1.5×10^{-5} M) with TNM (2.4×10^{-5} M) was completed within the mixing time of the stopped-flow apparatus (2.5 msec).

Oxidation by TNM of Reduced D-Amino Acid Oxidase —— The reaction of the reduced form of D-amino acid oxidase with TNM was used as a model for studying the reactivity of reduced flavin groups when combined to apoproteins. The reaction of the reduced enzyme (4.4×10^{-6} M) with TNM (1.6×10^{-4} M) was comparatively slow enough to be measured by the stopped-flow apparatus. The rapid absorbance change at 350 mμ was completed within 1 sec. This was

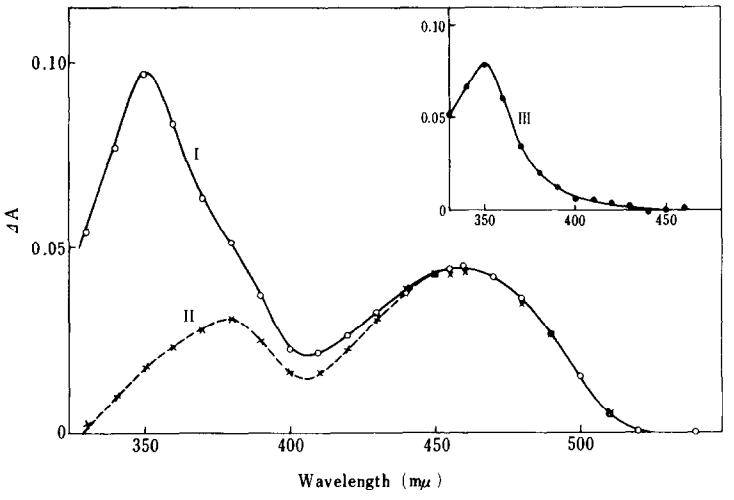

Fig. 1. Spectral change caused by the reaction of reduced D-amino acid oxidase with TNM. A solution of the reduced enzyme (8.8×10^{-6} M) was mixed with an anaerobic TNM solution (3.2×10^{-4} M) in a stopped-flow apparatus with a 2-mm cuvette. The final concentration of the reactants on mixing was reduced to half of the above values. The transmittance change at a number of wavelengths was recorded on a storage oscilloscope. Curve I: spectral change developed between zero time and 1 sec; curve II: difference spectrum between the oxidized and reduced forms of the enzyme; curve III: curve I minus curve II. The spectra shown in the figure were normalized for a light-path lengh of 1.0 cm.

followed by a very slow change*. The spectral change observed between zero time and 1 sec is shown by curve I in Fig. 1. The difference spectrum between the oxidized and reduced forms of the enzyme is shown by curve II. Subtraction of curve II from curve I yielded curve III, which is identical with the spectrum of the nitroform ion. It is, therefore, reasonable to ascribe the spectrum shown by curve I to the sum of the change due to the formation of nitroform ion and that due to the conversion of the reduced enzyme to the oxidized enzyme. On the other hand, the absorbance change within 1 sec of mixing TNM with the oxidized enzyme was negligible. These results indicate that the rapid spectral change between zero time and 1 sec is caused by the reaction of enzyme-bound

*The slow change could be attributed to the production of the nitroform ion either by the reaction of TNM with the enzyme protein or by the catalytic oxidation of D-arginine coupled to the reduction of TNM. The latter occurs due to the presence of a small excess amount of the substrate, D-arginine, used to obtain the reduced enzyme (see the next subsection).

Table I

Stoichiometry of the reaction of $FMNH_2$ with TNM

TNM added	FMN formed	nitroform ion formed	NO_2^- formed
160 mμmoles	99 mμmoles	105 mμmoles	105 mμmoles
64	64	65	61

To 3.0 ml of $FMNH_2$ solution (3.3×10^{-5} M), 10 or 4 μl of ethanolic TNM solution (1.6×10^{-2} M) was added anaerobically. The reaction was performed in 0.017 M sodium pyrophosphate buffer (pH 8.3) at 20°C. Nitroform ion was determined spectrophotometrically ($\varepsilon_{350} = 14{,}400\ M^{-1}\ cm^{-1}$). At 350 mμ, contribution of the absorbance change due to the oxidation of $FMNH_2$, was estimated from the absorbance change at 450 mμ, where contribution of the absorbance change due to the formation of nitroform ion was negligible. Determination of NO_2^-, see text.

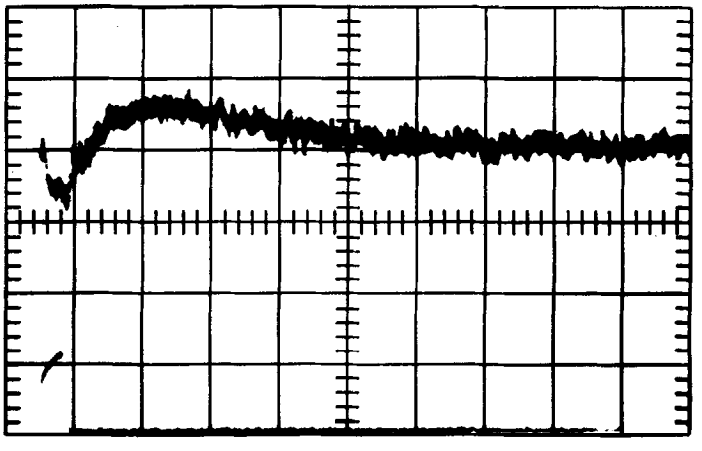

Fig. 2. Transient appearance of the semiquinoid enzyme in the reaction of reduced D-amino acid oxidase with TNM. The conditions were similar to those specified in Fig. 1. The concentration of the enzyme was 1.8×10^{-5} M (before mixing). The absorbance change was recorded at 520 mμ. The upper trace shows the reaction signal. Vertical scale, A = 0.00089 per major division. Horizontal scale, 100 msec per major division. The lower trace indicates the flow velocity.

flavin *per se*, but not by the reaction with $FADH_2$ released from the enzyme modified by TNM.

To examine the reaction in more detail, the time course of the absorbance changes at different wavelengths (330 to 540 mμ) was recorded. It was found

that they are not identical. The change at 455 mμ was nearly exponential, but the reaction trace at 520 mμ showed a transient and small increase in absorbance, as shown in Fig. 2. The change may indicate the transient appearance of a semiquinoid enzyme, which has considerable absorption at 520 mμ when compared to the oxidized and reduced enzyme (11). This was not the case with the reaction of the reduced enzyme with O_2.

If the oxidation of a reduced flavin (FH_2) by TNM occurred by a transfer of two electrons, TNM would decompose to form equimolar amounts of nitroform ion and NO_2^-.

$$FH_2 + C(NO_2)_4 = F + C(NO_2)_3^- + NO_2^- + 2H^+ \qquad (1)$$

This may be the case for the reaction of $FMNH_2$ with TNM. However, the evidence presented above suggests that the reaction of the reduced enzyme with TNM includes the formation of the semiquinoid enzyme (FH°). For this reaction, the equation may be written as,

$$FH_2 + C(NO_2)_4 = FH^\circ + C(NO_2)_3^- + NO_2 + H^+ \qquad (2)$$

$$FH^\circ + C(NO_2)_4 = F + C(NO_2)_3^- + NO_2 + H^+ \qquad (3)$$

If reactions 2 and 3 were the only processes in the oxidation of the reduced enzyme, the molar ratio of nitroform ion to the enzyme should be 2. Since the observed ratio was 1.3 as calculated from Fig. 1, it is reasonable to consider that a 2-electron transfer reaction occurs in parallel with reactions 2 and 3, or that NO_2 formed in reaction 2 partially oxidizes the semiquinoid enzyme.

Catalytic Oxidation of Substrate in the Presence of TNM —— The formation of nitroform ion in the catalytic oxidation of substrate in the presence of TNM was observed by measuring increase in absorbance at 350 mμ. When a solution of D-arginine plus TNM was mixed with the enzyme (2.3×10^{-6} M), the rate of formation of nitroform ion was dependent upon the concentration of D-arginine as shown in Fig. 3. In the case of D-alanine (1.0×10^{-3} M), however, the rate was nearly same as in the control experiment carried out in the absence of the substrate (see Fig. 3).

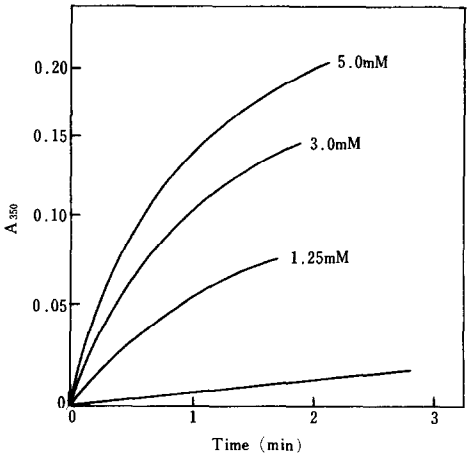

Fig. 3. The formation of nitroform ion during oxidation of D-arginine by D-amino acid oxidase. An aerobic solution of the oxidized enzyme (2.3×10^{-6} M, final concentration) and D-arginine solution (final concentration indicated in the figure), containing TNM (1.6×10^{-4} M, final concentration) were mixed in a stopped-flow apparatus with a 2-mm cuvette. The transmittance change at 350 mμ was recorded. The transmittance coordinate was converted to absorbance. The lowest curve shows the trace on mixing the oxidized enzyme and TNM in the absence of the substrate.

Table II

Effect of TNM on the oxygen consumption due to oxidation of D-alanine or D-arginine by D-amino acid oxidase

substrate	D-amino acid oxidase	TNM	rate (sec^{-1})
D-alanine (1.0×10^{-2} M)	4.8×10^{-7} M	nil	3.2
		1.6×10^{-4} M	3.1
D-arginine (5.0×10^{-3} M)	1.5×10^{-6} M	nil	0.64
		8.1×10^{-5} M	0.20
		1.6×10^{-4} M	0.13

The initial velocity of the oxygen consumption due to oxidation of substrate was measured in the presence or absence of TNM. The total volume of the reaction mixture was 3.0 ml. The rate was expressed as moles of O_2 consumed /sec/mole of enzyme-bound FAD.

When oxygen consumption was measured in the reaction with D-arginine, it was found to be remarkably depressed with increasing concentration of TNM,

indicating competition between O_2 and TNM. In contrast, the oxygen consumption due to the reaction with D-alanine was scarcely depressed (Table II).

These observations can be explained by the difference in the mode of reaction of the enzyme with the two substrates. The enzyme shuttles between the oxidized and reduced forms with D-arginine as substrate (12), whereas with D-alanine as substrate, it shuttles between the oxidized form and the purple intermediate which is slowly converted to the reduced form (13,14). The above result can therefore be interpreted to mean that the purple intermediate has little or no reactivity with TNM.

Evidence presented in this paper suggests that the reaction of reduced D-amino acid oxidase with TNM involved the semiquinoid enzyme. Although the enzyme mediates a 2-electron transfer in the reaction with O_2, a 1-electron transfer with acceptors such as TNM can also occur. The present observations highlight the differences in the interaction of enzyme-bound and free flavins as well as the occurrence of different types of electron transfer catalyzed by flavoproteins with a variety of acceptors.

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