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A STOPPED-FLOW STUDY OF THE REACTION BETWEEN MERCURIC REDUCTASE AND NADPH

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The reaction of the FAD-containing enzyme, mercuric reductase, with NADPH has been studied by stopped-flow kinetic methods at 25 °C, pH 7.3. The results suggest that the reaction involves at least three steps. The first step is very rapid and is essentially complete within the dead time of the stopped-flow apparatus. This step is associated with decreasing absorbances at 340 nm (NADPH) and 455 nm (FAD), whereas there is little formation of the absorbance at 530 nm characterizing 2-electron-reduced enzyme subunits (EH_2). The second step involves an increase of the absorbance at 530 nm. The third step results in an increase of the intensity of the long-wavelength band and a change of its shape. A second equivalent of NADPH per FAD is required for this step. It is proposed that the product is an EH_2-NADPH complex. In addition to these rapid steps, slow absorbance changes are also observed.

Mercury(II)-resistant bacteria can produce a plasmid-encoded enzyme, mercuric reductase (reduced NADP: mercuric ion oxidoreductase), catalyzing the reduction of Hg^{2+} to metallic mercury, Hg^0 (1): $Hg^{2+} + NADPH \rightarrow Hg^0 + NADP^+ + H^+$.

Mercuric reductase from *Pseudomonas aeruginosa* carrying the plasmid pVS1 is dimeric (M_r = 123000) and contains one molecule of FAD and a redox-active disulfide per subunit (2). The enzyme requires the presence of a thiol compound for sustained turnover (2-4).

The 2-electron reduction of mercuric reductase subunits results in the formation of a new, broad absorption band centered near 530 nm, while the bands characteristic of oxidized FAD change rather little. These spectroscopic properties are strikingly similar to those shown by lipoamide dehydrogenase and glutathione reductase, which are FAD-containing enzymes catalyzing electron transfers between pyridine nucleotides and disulfide substrates (5). It has been proposed that, in these enzyme as well as in mercuric reductase, the FAD moiety remains in the oxidized state while the active-site disulfide is reduced.

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The long-wavelength band is though to result from a charge-transfer interaction between FAD and one of the active-site SH-groups (5).

MATERIALS AND METHODS

Enzyme preparation and assay. *Pseudomonas aeruginosa*, strain PAO 9501, carrying the plasmid pVS1 (kindly donated by Dr. Simon Silver, Washington University, St. Louis, MO, U.S.A.) was grown (2 x 15 L) at 37 °C in a medium containing 13 g/L Nutrient Broth (Oxoid Ltd, London, England), 1 g/L Bacto-Tryptone (Difco Laboratories, Detroit, MI, U.S.A.), 1 mg/L thiamine, 10 mM citric acid, 0.8 mM $MgSO_4$, 57 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$. The medium also contained 5 μM $HgCl_2$ to induce mercuric reductase. After 30 min, $HgCl_2$ was added to a final concentration of 35 μM. At late logarithmic phase, the cells were harvested in a Sharples centrifuge and resuspended in 150 mL of 50 mM potassium phosphate buffer, pH 7.3. This buffer was used throughout the preparation. The cells were disrupted in a French press at 275 atm passing the suspension twice through the cell. DNA, which made the suspension very viscous, was degraded by the addition of 1 mg deoxyribonuclease I (Leningrad United Meat Production Industries), 1 mM EDTA, 0.1 mM dithiothreitol (Sigma Chemical Company, St. Louis, MO, U.S.A.), and 10 mM magnesium acetate. The suspension was incubated at 37 °C for 10 min and then heated for 20 min in a water bath kept at 61 °C. Cell debris and precipitated proteins were removed by centrifugation at 60000 x g for 12 h. The supernatant solution was applied to an Orange A Matrex (Amicon, Lexington, MA, U.S.A.) column (5 x 50 cm) and eluted with NADPH (Sigma) as described by Fox and Walsh (2). The pooled and concentrated enzyme-containing fractions were dialyzed against 2 M KBr followed by 50 mM potassium phosphate buffer, pH 7.3, to remove $NADP^+$ bound to the enzyme (2). Throughout the preparation the enzyme was assayed following the volatilization of Hg(0) using radioactive $^{203}HgCl_2$ (New England Nuclear, Dreieich, W. Germany) as substrate as described by Schottel (3). Enzyme concentrations were estimated by flavin absorbance using a millimolar absorption coefficient at 455 nm of 11.3 $mM^{-1}cm^{-1}$ for enzyme-bound FAD (2).

Absorption spectra. All absorption coefficients are calculated on the basis of FAD concentration. Routine spectra were recorded on a Perkin-Elmer 320 spectrophotometer. For rapid recording of spectra a Hewlett-Packard 8450 UV/VIS spectrophotometer was employed. Using a mixing device with two interconnected 1 mL syringes, enzyme and NADPH solutions were mixed directly into the cuvette. Spectra were recorded at room temperature between 300 and 700 nm every 2 s for 40 s. The first spectrum was recorded approximately 4 s after mixing.

Stopped-flow measurements. Most measurements were made aerobically at 25 °C with an Aminco stopped-flow apparatus connected to a Hewlett-Packard 9835A desk computer as described by Simonsson and Lindskog (6). Anaerobic stopped-flow measurements were performed at 25 °C with a Durrum apparatus as described by Andréasson et al. (7,8). Apparent first-order rate constants were calculated from the relationship $k = d(\ln| A-A_{eq}|)/dt$ where A and A_{eq} are the absorbances at time t and at equilibrium, respectively.

RESULTS AND DISCUSSION

Absorption spectra and stoichiometry. Spectrophotometric titrations of mercuric reductase with NADPH under aerobic conditions did not yield a clear estimate of the stoichiometry of the reaction. It was later found that, in addition to rapid spectral changes occurring in the milliseconds time range,

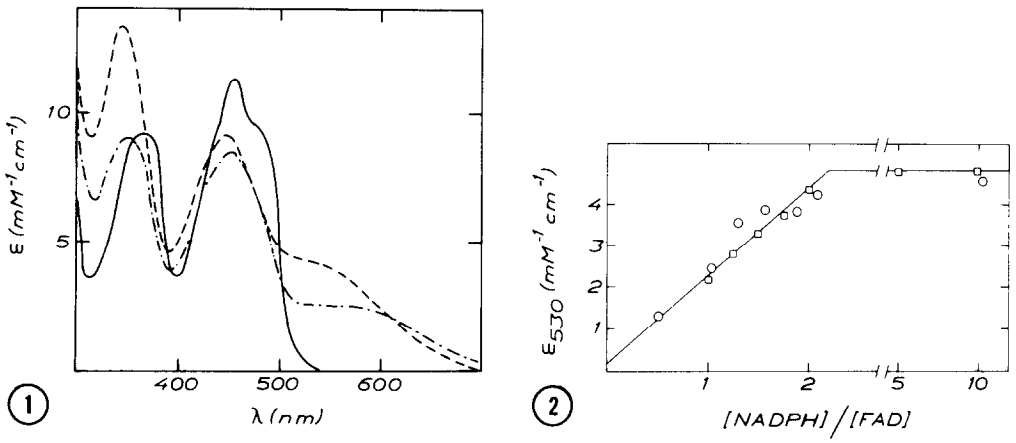


Fig. 1. Effects of NADPH on the absorption spectrum of mercuric reductase. Spectra were recorded 4 s after mixing. Molar ratio NADPH/FAD: (——), 0; (— · —), 1.0; (---), 2.0. 50 mM potassium phosphate buffer, pH 7.3. Aerobic conditions. Room temperature. Enzyme-bound FAD concentration, 19.4 μM.

Fig. 2. Titration of mercuric reductase with NADPH measured at 530 nm. (o), millimolar absorption coefficient, ε, calculated from spectra recorded 4 s after mixing. Conditions as in Fig. 1. (□), ε obtained in stopped-flow experiments after decay of rapid transient, 50-100 ms after mixing. Aerobic conditions; 50 mM potassium phosphate buffer, pH 7.3; enzyme-bound FAD concentration, 8.7 μM; 25 °C.

there are also slow changes that are not complete within 10 min. These slow changes take place under anaerobic conditions as well. To characterize the spectral changes associated with the rapid reactions we performed another titration recording the spectra within 4 s after mixing. A few of these spectra are shown in Fig. 1. Two important observations can be made. First, more than one equivalent of NADPH per FAD is required before the band centered near 530 nm is fully formed. Second, there are no isosbestic points throughout the titration showing that at least three spectral species are involved. At NADPH/FAD ratios below one, the long-wavelength band is centered near 570 nm, while it is gradually shifted towards 530 nm as the NADPH/FAD ratio increases.

Fox and Walsh (2) titrated mercuric reductase with sodium dithionite. They concluded that only one dithionite/FAD is needed to obtain a maximal intensity at 540 nm. We have repeated the dithionite titration under carefully controlled anaerobic conditions and confirm this 1/1 stoichiometry.

Fig. 2 shows the millimolar absorption coefficient at 530 nm as a function of the NADPH/FAD ratio. Amplitudes of the rapid transients observed

in stopped-flow experiments are also included. These results clearly show a 2/1 stoichiometry between NADPH and FAD. We suggest that the species obtained with two or more equivalents of NADPH per FAD is a complex between 2-electron-reduced enzyme subunits and NADPH, EH_2-NADPH. An analogous complex is found in the reaction between NADPH and glutathione reductase (9).

The nature of the species having the long-wavelength band centered near 570 nm is not so clear. However, its formation is associated with $\Delta\varepsilon_{340} = -4.5$ $mM^{-1}cm^{-1}$ indicating that most, if not all, of one equivalent of NADPH/FAD has been oxidized. Therefore, it seems likely that the 570 nm band represents species on the EH_2 oxidation level, possibly an EH_2-$NADP^+$ complex.

Kinetics of rapid absorbance changes. Various concentrations of NADPH were mixed with mercuric reductase in a stopped-flow apparatus at 25 °C, pH 7.3. The results obtained under aerobic or anaerobic conditions were not significantly different. The observed transients were only marginally affected by the addition of 1 mM cysteine to the medium. The results described below were obtained without added thiol compound.

The observed increases of A_{530} gave linear first-order plots. The apparent rate constants are given in Table I. While the total amplitudes increase with increasing NADPH/FAD ratio (Fig. 2), the rate constants decrease. Above NADPH/FAD = 2 no significant further changes are observed. Extrapolating the exponential curves to time zero we find that the reaction at NADPH/FAD = 1 appears to start near $A_{530} = 0$, while the reaction at higher ratios appears to start at

Table I. Apparent first-order rate constants for the reaction between mercuric reductase and NADPH observed at 530 nm.

Molar ratio NADPH/FAD	Rate constant (s^{-1})
1.0	140
1.25	123
1.5	97
1.75	82
2.0	63
5.0	51
10.0	56

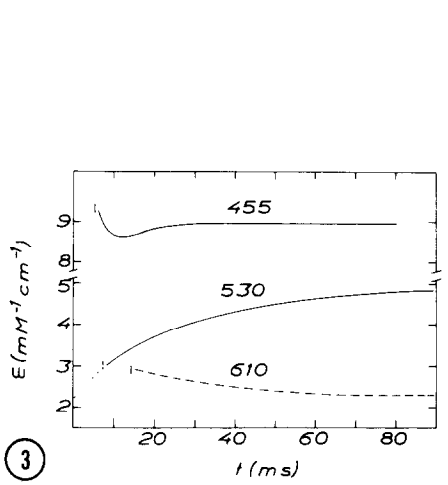
Reaction medium, 50 mM potassium phosphate buffer, pH 7.3; temperature, 25 °C; FAD concentration, 8.7 μM.

larger values of A_{530} (Fig. 3). This somewhat puzzling behaviour indicates that at least two processes are involved, a fast process which may or may not depend on the NADPH concentration, and a slower process with k near 50 s^{-1} which requires a second equivalent of NADPH. The observed traces at NADPH/FAD ratios between 1 and 2 fit with the sum of two exponential processes with an increasing amplitude of the slower one as the NADPH concentration increases. If we assume that the product of the fast process is an EH_2-$NADP^+$ complex, the slower process might represent the displacement of $NADP^+$ by NADPH. The rate of this step might be limited by the dissociation of $NADP^+$.

The major fractions of the absorbance changes at 340 nm and 455 nm take place within the dead time of the stopped-flow apparatus. At an NADPH/FAD ratio of 1, the initial, rapid decrease of A_{455} is followed by a very small increase ($\Delta\varepsilon$ = 0.1 $mM^{-1}cm^{-1}$) concurrently with the increase of A_{530}. Thus, A_{455} shows a minimum about 12 ms after mixing. This observation is evidence that also under these conditions, when A_{530} appears to behave as a simple exponential, two processes are occurring, one too fast to be measured and one with k approximately 140 s^{-1}.

With an excess of NADPH, it was found that the slowest step ($k \approx 50$ s^{-1}) is associated with a decrease of A_{340}, a small decrease of A_{610}, and an increase of A_{455} in addition to the increase of A_{530}. Some of these patterns are shown in Fig. 3. The different time courses of A_{530} and A_{610} indicate that the slowest step is associated with a change of the shape of the long-wavelength band.

Our kinetic results can be interpreted in terms of a reaction sequence similar to that proposed by Huber and Brandt (10) for the reaction between NADPH and glutathione reductase: A → B → C → D, where A represents oxidized enzyme, B represents a species formed within the dead time, C represents the end product at NADPH/FAD = 1, and D represents the putative EH_2-NADPH complex. We refrain from a more detailed interpretation until more extensive data have been obtained.



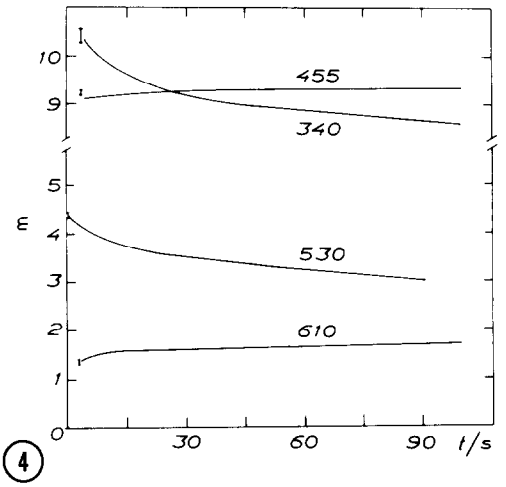


Fig. 3. Stopped-flow measurements of rapid absorbance changes in the reaction between mercuric reductase and an excess of NADPH. Millimolar absorption coefficients, ε, at 455, 530, and 610 nm. The 455 nm experiment was performed anaerobically with a molar ratio NADPH/FAD = 2.2. The experiments at 530 and 610 nm were performed aerobically with NADPH/FAD ratios of 5.7 and 6.1, respectively. The 530 nm curve has been extrapolated to time zero (dotted line). All experiments were performed in 50 mM potassium phosphate buffer, pH 7.3 at 25 °C. Enzyme-bound FAD concentrations between 8 and 10 μM. Error bars represent maximal noise levels.

Fig. 4. Stopped-flow measurements of slow absorbance changes in the reaction between mercuric reductase and NADPH. Only the initial phase is shown. Millimolar absorption coefficient, ε, at 340, 455, 530, and 610 nm at NADPH/FAD ratios near 2. All experiments were performed in 50 mM potassium phosphate buffer, pH 7.3, 25 °C. Enzyme-bound FAD concentrations were between 8 and 10 μM. Error bars represent maximum noise levels.

Slow absorbance changes. Slow absorbance changes observed at an NADPH/FAD ratio of about 2 are shown in Fig. 4. They are associated with a red shift of the long-wavelength band. At an NADPH/FAD ratio of 1 the slow changes are in the direction towards the spectrum of oxidized enzyme (data not shown). These changes do not follow a simple first-order pattern. They are far too slow to be involved in catalytic turnover ($k_{cat} \approx 15\ s^{-1}$ (2,4)).

The rates of the slow changes are virtually identical in aerobic and in anaerobic solutions. Although the nature of these changes is unclear, it is possible that they, to some extent, represent interconversions between species on the EH_2 level and/or a partial disproportionation to E and EH_4 as proposed by Wilkinson and Williams (11) for similar, slow reactions in the *E. coli* lipoamide dehydrogenase system. However, we observe a continuous decrease of

A_{340} at all studied NADPH concentrations, and this indicates that a net oxidation by traces of O_2 is also taking place.

In the presence of a large excess of NADPH there are no significant, slow changes of the visible spectrum of mercuric reductase. Thus, as long as there is a sufficient supply of NADPH to keep the enzyme as an EH_2-NADPH complex, the thiolate-FAD charge interaction appears to be stable.

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