Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 518-523

REACTIVITY OF AN FAD-DEPENDENT OXYGENASE WITH FREE FLAVINS: A NEW MODE OF UNCOUPLING IN FLAVOPROTEIN OXYGENASES

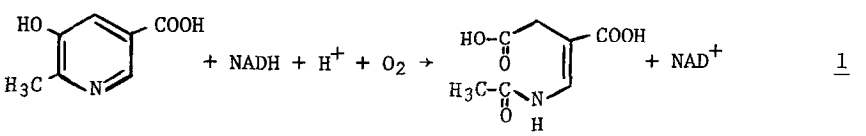
Ganesh M. Kishore and Esmond E. Snell

Departments of Microbiology and Chemistry
The University of Texas, Austin, Texas 78712

Received February 9,1979

SUMMARY: Conversion of 2-methyl-3-hydroxypyridine-5-carboxylic acid (Cpd I) to α-(N-acetylaminomethylene)succinic acid (Cpd A) is catalyzed by an FAD protein, Cpd I oxygenase (Sparrow, et al., J. Biol. Chem. [1969] 244, 2590-2600) according to the equation: Cpd I + O_2 + NADH + H^+ → Cpd A + NAD^+. When free FAD, FMN or riboflavin is added to reaction mixtures, oxidation of NADH remains dependent on presence of oxygenase and Cpd I, but is partially uncoupled from the oxygenation of Cpd I. Under these conditions, free reduced flavins appear in solution, as shown by their ability to reduce cytochrome c. These effects are not due to an increased rate of NADH oxidation. Such uncoupling may lead to appearance of artifactual species of activated oxygen or flavin that play no intermediate role in the oxygenase reaction.

INTRODUCTION: Many studies of the mechanism of action of flavoprotein oxygenases aimed at identifying the activated oxygen species that participate in the oxygenation reaction have appeared during recent years (1, 2). We previously reported (3) the purification from Pseudomonas MA-1, grown on pyridoxine as sole source of carbon, of an FAD-dependent oxygenase that catalyzes reaction 1:



Cpd I Cpd A 1

Since reaction 1 is one of a very few ring cleavage reactions (4) reported to be catalyzed by flavoproteins, it is important to study its mechanism. During these investigations, we observed that addition of flavins could reduce the extent to which NADH oxidation was coupled (2) to oxygenation of Cpd I. To our

*This work was supported in part by Grant F-714 from the Robert A. Welch Foundation and by Grants AM 19898 and AI 13940 from the National Institutes of Health.

0006-291X/79/060518-06$01.00/0

knowledge, this constitutes the first report of uncoupling in flavooxygenases induced by free riboflavin and its coenzymes. Since several species of flavins and of activated oxygen arise during autoxidation of reduced flavins, electron transfers between flavooxygenases and free flavins (or other flavoproteins) similar to those described here may confuse the search for intermediates in mechanistic studies of oxygenase action.

MATERIALS AND METHODS: NADH was from Boehringer Mannheim. Cpd I was isolated from culture fluids of Pseudomonas MA-1 grown on pyridoxine and purified by Dowex-1-formate chromatography and recrystallization (5). Riboflavin, FAD and FMN were from Sigma Chemical Company. Cpd I oxygenase was purified by the method of Sparrow, et al. (3). 10 mM dithiothreitol was used in place of 0.1% β-mercaptoethanol in the above purification procedure. The apoenzyme of Cpd I oxygenase was prepared by the method of Sparrow, et al. (3) with omission of bovine serum albumin and substitution of dithiothreitol for β-mercaptoethanol.

Assay. A standard reaction mixture contained 0.2 μmoles of Cpd I, 0.2 μmoles of NADH and 0.02 nmoles of enzyme (based on a subunit mol. wt. of 83,000) in 1 ml of 0.1 M potassium phosphate, pH 7.0 at 25°C. The reaction was started by adding the enzyme and was followed by measuring absorbance changes at 326 and 350 nm in a Cary 118C spectrophotometer. Cpd I has an extinction of 650 M^{-1} cm^{-1} at 350 nm and 4,400 M^{-1} cm^{-1} at 326 nm, while the extinction of NADH at these wavelengths is 5,500 M^{-1} cm^{-1} and 5,300 M^{-1} cm^{-1}, respectively. If ΔA is the observed absorbance change, the extent of NADH oxidation and of Cpd I oxygenation in a single reaction mixture can be calculated from the simultaneous equations:

$$\Delta A_{326} = 4400 \cdot \Delta C_{\text{Cpd I}} + 5300 \cdot \Delta C_{\text{NADH}}$$

$$\Delta A_{350} = 650 \cdot \Delta C_{\text{Cpd I}} + 5500 \cdot \Delta C_{\text{NADH}}$$

where ΔC is the change in concentration.

RESULTS: In Fig. 1, the molar ratio of NADH oxidized to Cpd I utilized is plotted as a function of concentration of added flavins. This ratio changes from 1.18 in the absence of any added flavin to a maximum of 3.28 in the presence of FMN or riboflavin. Several explanations to account for the increased consumption of NADH were examined. The possibility that a flavin-dependent NADH oxidase is present in the enzyme preparation was excluded, since addition of flavins to either the apo- or the holoenzyme preparation did not lead to NADH oxidation in the absence of Cpd I. Furthermore, the enzyme migrates as a

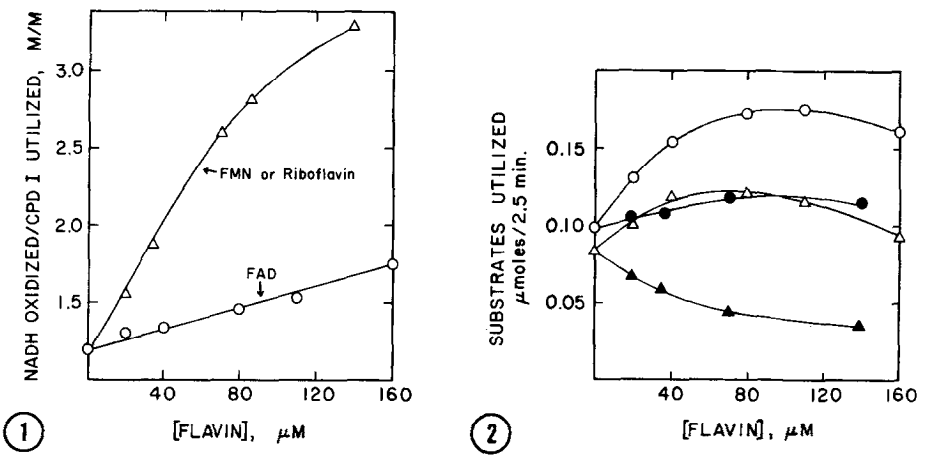


Fig. 1 Effect of free flavins on the molar ratio of NADH oxidized to Compound I oxygenated during catalysis of Reaction 1 by Compound I oxygenase. Assays were performed as described in "Methods" in the presence of 1.66 μg/ml of oxygenase (0.02 μM subunit concentration).

Fig. 2 Comparative rates of oxidation of NADH (O-O, ●-●) and of Compound I (Δ-Δ, ▲-▲) in the presence of FAD (O-O, Δ-Δ) or FMN (●-●, ▲-▲). Riboflavin and FMN gave almost identical effects. Assay procedures are described in Fig. 1.

single band on polyacrylamide gel electrophoresis, which argues against the presence of any major contamination by another enzyme.

A second possibility is that NADH is oxidized at a higher rate in the presence of free flavins and that the oxygenation rate cannot be elevated to a commensurate level. Although the rate of NADH oxidation does increase significantly in the presence of free FAD, it does not do so with FMN or riboflavin (Fig. 2). Since the degree of uncoupling is least with FAD (Fig. 1), an increased rate of NADH oxidation does not explain the increased NADH consumption.

A third possibility is that added flavins (abbreviated as Fl) inhibit the oxygenation reaction. Kinetic studies yet to be published of the action of Cpd I oxygenase are consistent with the formulation shown in reactions (a) and (b) below, where E is the apooxygenase. Although inhibition due to competition of Fl with Cpd I for the Cpd I binding site on the enzyme is very unlikely, a more direct possibility is that bound Cpd I and free Fl compete for reduced $FADH_2$ in the active site of the enzyme as indicated by reaction (b) vs. (c) below.

$$\text{(a)}\quad \text{NADH} + \underset{\text{Cpd I}}{\text{E-FAD}} \rightleftarrows \underset{\text{Cpd I}}{\text{E-FADH}_2} + \text{NAD}^+$$

$$\text{(b)}\quad \text{E}\begin{matrix}\nearrow \text{FADH}_2 \\ \searrow \text{Cpd I}\end{matrix} + \text{O}_2 \rightarrow \text{E}\begin{matrix}\nearrow \text{FAD} \\ \searrow \text{Cpd I}\end{matrix} + \text{Cpd A}$$

$$\text{(c)}\quad \text{E}\begin{matrix}\nearrow \text{FADH}_2 \\ \searrow \text{Cpd I}\end{matrix} + \text{Fl}^{(\text{ox})}_{\text{free}} \rightleftarrows \text{E}\begin{matrix}\nearrow \text{FAD} \\ \searrow \text{Cpd I}\end{matrix} + \text{Fl}^{(\text{red})}_{\text{free}}$$

$$\text{(d)}\quad \text{Fl}^{(\text{red})}_{\text{free}} + \text{O}_2 \rightarrow \text{Fl}^{(\text{ox})}_{\text{free}} + \text{H}_2\text{O}_2$$

In this case, reduced flavins should appear in the reaction mixture. Since cytochrome c is reduced non-enzymatically by reduced flavin (6), but is reduced very poorly by Cpd I oxygenase (Table I), it was chosen to test for the presence of free reduced flavins in solution. Cytochrome c reduction is stimulated greatly in reaction mixtures that contain added flavins, thus demonstrating that reduced flavins are formed in these solutions (Table I). The rate of reduction of cytochrome c with added FAD is only half that obtained at an equivalent concentration of added FMN or riboflavin.

DISCUSSION: Many metabolic reactions involve oxido-reduction reactions between bound FAD molecules on different interacting flavoproteins or between FAD and FMN bound to the same enzyme protein (7, 8). Passage of electrons between flavoproteins and free flavins during the aerobic turnover of these enzymes has been detected much less frequently. Morell (9) demonstrated that in the presence of substrate, the tightly bound flavin of xanthine oxidase slowly reduced added riboflavin, FMN or FAD, whereas glucose oxidase and D-amino acid oxidase were unable to do so. Hager, et al. (10) demonstrated in L. delbrueckii that free riboflavin could carry electrons between the two FAD proteins, pyruvic and lactic dehydrogenases.

There are a few reports (11-13) of the rapid reduction of free flavins by flavoprotein oxygenases. Ohta, et al. (11) demonstrated that orcinol hydroxylase reduced added FAD anaerobically and Neujahr and Kjeller (12) recently demonstrated a similar reaction with phenol hydroxylase. Neither group mea-

Table. I. Effects of Free Flavins on the Rates of Cytochrome c Reduction in the Presence of Compound I Oxygenase.

Additions to reaction mixture Additions (nmoles/ml)	Cytochrome c reduced nmoles/min
None	0.001
FAD (40)	0.005
Oxygenase (0.04)	1.5
Oxygenase (0.04) + FAD (40)	30.0
Oxygenase (0.04) + FMN (40)	63.7
Oxygenase (0.04) + Riboflavin (40)	60.0

The reaction mixtures contained in all cases 0.2 µmole of Compound I, 0.2 µmole of NADH and 0.08 µmole of cytochrome c in 1.0 ml of air-saturated 0.1 M potassium phosphate buffer, pH 7.0. Cytochrome c reduction in the reaction mixtures was followed to completion at 25° in the Cary spectrophotometer at 550 nm.

sured the tightness of coupling of NADH oxidation to substrate hydroxylation under aerobic conditions in reaction mixtures containing free FAD.

The present data show that NADH oxidation is uncoupled from substrate oxygenation by free flavins and that this uncoupling results from an electron transfer from reduced enzyme to the free flavins, and not from an amplification of the rate of NADH oxidation. If all three reduced flavins react with cytochrome c at equal rates, the fact that the extent of uncoupling with FMN or riboflavin is almost twice that with FAD indicates either that the accessibility of free FAD to the reduced active site must be only about half that of FMN or riboflavin, or that the enzyme rapidly reoxidizes free reduced FAD. It is significant in this connection that neither FMN nor riboflavin can replace FAD as cofactor for Cpd I oxygenase ([3] and unpublished data).

FAD is frequently added to reaction mixtures during mechanistic studies of FAD-dependent oxygenase reactions (12). As shown here for Cpd I oxygenase, this may result in formation of free $FADH_2$ and hence the non-enzymatic formation of various species of activated oxygen or flavins by reactions such as (d) shown above. Under these circumstances, the assumption that such species are involved in the mechanism of the oxygenation reaction *per se* may be erroneous. To illustrate this point, we have demonstrated reduction of cytochrome c by Cpd I oxygenase in the presence of its substrates and FAD. Reduction of cyto-

chrome c under these conditions is partly inhibited by superoxide scavenging radicals (unpublished data), indicating that the cytochrome is reduced in part by superoxide ion. Similarly, partial uncoupling of Cpd I oxygenation from NADH oxidation was observed when both superoxide dismutase and FAD were added to Cpd I oxygenase reaction mixtures. In the absence of knowledge concerning electron transfer between flavooxygenases and free flavins, one would be tempted to draw the unjustified conclusion that superoxide ions are involved in the oxygenation reaction.

REFERENCES

1. Massey, V. and Hemerich, P. (1975) in "The Enzymes" (Boyer, P. D., ed.) XII B, pp. 191-252, Academic Press, N. Y.
2. Flashner, M. S. and Massey, V. (1974) in "Molecular Mechanisms of Oxygen Activation" (Hayaishi, O., ed.) pp. 245-283, Academic Press, N. Y.
3. Sparrow, L. G., Ho, P. P. K., Sundaram, T. K., Zach, D., Nyns, E. J. and Snell, E. E. (1969) J. Biol. Chem. 244, 2590-2600.
4. Hayaishi, O., Nozaki, M. and Abbott, M. T. (1975) in "The Enzymes" XII, part B, p. 119, Third Edition (Boyer, P. D., ed.) Academic Press, N. Y.
5. Burg, R. W., Rodwell, V. W. and Snell, E. E. (1960) J. Biol. Chem. 235, 1164-1169.
6. Singer, T. P. and Kearney, E. B. (1950) J. Biol. Chem. 183, 409-429.
7. Faeder, E. J., Davis, P. S. and Siegel, L. M. (1974) J. Biol. Chem. 249, 1599-1609.
8. Vermillion, J. L. and Coon, M. J. (1978) J. Biol. Chem. 253, 8812-8819.
9. Morrell, D. B. (1952) Biochem. J. 51, 657-666.
10. Hager, L. P., Geller, D. M. and Lipmann, F. (1954) Fed. Proc. 13, 734.
11. Ohta, Y., Higgins, I. J. and Ribbons, D. W. (1975) J. Biol. Chem. 250, 3814-3825.
12. Neujahr, H. Y. and Kjeller, K. G. (1978) J. Biol. Chem. 253, 8835-8841.
13. Hosokowa, K. and Stanier, R. Y. (1966) J. Biol. Chem. 241, 2453-2460.