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Studies of the Control of Luminescence in *Beneckea harveyi:* Properties of the NADH and NADPH:FMN Oxidoreductases†

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ABSTRACT: Highly purified NADH and NADPH:FMN oxidoreductases from *Beneckea harveyi* have been characterized with regard to kinetic parameters, association with luciferase, activity with artificial electron acceptors, and the effects of inhibitors. The NADH:FMN oxidoreductase exhibits single displacement kinetics while the NADPH:FMN oxidoreductase exhibits double displacement or ping-pong kinetics. This is consistent with the formation of a reduced enzyme as an intermediate in the reaction catalyzed by the NADPH:FMN oxidoreductase. Coupling of either of the oxidoreductases to the luciferase reaction decreases the apparent K_ms for NADH, NADPH, and FMN, supporting the suggestion of a complex between the oxidoreductases and luciferase. The soluble oxidoreductases are more efficient in producing light with luciferase than is a NADH dehydrogenase preparation obtained from the membranes of these bacteria. The soluble enzymes use either FMN or FAD as substrates for the oxidation of reduced pyridine nucleotides while the membrane NADH dehydrogenase is much more active with artificial electron acceptors such as ferricyanide and methylene blue. FMN and FAD are very poor acceptors. The evidence indicates that neither of the soluble oxidoreductases is derived from the membranes. Both enzymes are constitutive and do not depend on the synthesis of luciferase.

The production of light by extracts of luminescent bacteria is due to the interaction of reduced flavin mononucleotide, $FMNH_2$,[1] a long chain aldehyde, molecular oxygen, and a specific enzyme, luciferase (Strehler & Cormier, 1953; McElroy et al., 1953; Becvar & Hastings, 1975; Hastings & Wilson, 1976). Reaction 1 describes the overall process for light emission.

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\nu \quad (1)$$

The generation of reduced FMN is dependent on the presence of specific pyridine nucleotide oxidoreductases which can use either NADH or NADPH as electron donors (reaction 2).

$$NAD(P)H + H^+ + FMN \rightarrow NAD(P)^+ + FMNH_2 \quad (2)$$

In *Photobacterium fischeri* there appears to be only one oxidoreductase which uses either NADH or NADPH (Duane & Hastings, 1975). *Beneckea harveyi* has been shown to have two distinct oxidoreductases, one specific for NADH and another specific for NADPH (Gerlo & Charlier, 1975). These enzymes use soluble FMN as a substrate unlike many other flavin utilizing enzymes which contain the flavin as a tightly bound cofactor. Similar oxidoreductases have been found in *Photobacterium phosphoreum, Escherichia coli, Azotobacter vinelandii,* and *Clostridium perfringens* (Puget & Michelson, 1972).

We report here further characterization of the NADH and NADPH:FMN oxidoreductases from *B. harveyi* with regard to kinetics and their association with luciferase. The effect of artificial electron acceptors and various inhibitors on the activity of these two enzymes was also investigated. The properties of the soluble oxidoreductases were compared with the membrane-bound NADH dehydrogenase.[2]

Materials and Methods

Chemicals. NADH, NADPH, AMP, and dithiothreitol were obtained from Calbiochem. FAD, FMN, decanal, rotenone, dicoumarol, 2-heptyl-4-hydroxyquinoline *N*-oxide, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, Triton X-100, and DNase I were purchased from Sigma Chemical Co. Dichlorophenolindophenol, menadione, and methylene blue were from the Aldrich Chemical Co. 5,5′-Dithiobis(2-nitrobenzoic

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; $FMNH_2$ and FMN, reduced and oxidized riboflavin 5-phosphate; DCIP, dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoate; DNP, dinitrophenol.

[2] We will refer to the soluble enzymes which use FMN as a substrate as oxidoreductases and the membrane bound enzyme as NADH dehydrogenase.

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TABLE I: The Michaelis Constants for the NADH and NADPH:FMN Oxidoreductase.[a]

Assay	mol of luciferase/ mol of oxidoreductase	Enzyme	pH	K_ms (μM) NADH	NADPH	FMN
Spectrophotometric		NADH specific	8.5	12		1.0
		NADH specific	7.0	47		1.0
Spectrophotometric		NADPH specific	5.6		20	18
		NADPH specific	7.0		40	14
Coupled	600	NADH specific	7.0	5.0		0.35
	0.2	NADH specific	7.0	7.0		0.45
Coupled	600	NADPH specific	7.0		1.6	2.50
	0.2	NADPH specifc	7.0		2.0	1.5

[a] The K_ms were obtained spectrophotometrically by following the disappearance of NADH or NADPH by decrease in optical density at 340 nm due to oxidoreductase activity alone. The K_ms were obtained in the coupled assay by monitoring the maximal light intensity generated by luciferase and oxidoreductase.

acid) was from P-L Biochemicals, Inc., and *o*-phenanthroline came from the Fisher Scientific Co.

Bacteria. Beneckea harveyi strain no. 392 (Reichelt & Baumann, 1973) was obtained as described earlier (Jablonski & DeLuca, 1977). Frozen cells were used in the preparation of the NADH and NADPH:FMN oxidoreductases and luciferase. Freshly harvested cells, grown in synthetic salt water media (Farghaly, 1950) with the addition of 5 g of peptone and 3 g of yeast extract (Difco) per L, were used for the preparation of the membranes.

Enzyme Preparation. The purification of the NADH and NADPH:FMN oxidoreductases has been described previously by Jablonski & DeLuca (1977). The preparations used in this study had specific activities of 36 μmol of NADH oxidized per min per mg of protein at pH 8.5 and 70 μmol of NADPH oxidized per min per mg of protein at pH 5.6 for the NADH and NADPH:FMN oxidoreductase, respectively. These specific activities are somewhat higher than those previously reported at pH 7.0 (Jablonski & DeLuca, 1977).

Luciferase was purified according to the method of Gunsalus-Miguel et al. (1972) with two additional steps: chromatography on a Sephadex G-100 column, 2.5 cm × 80 cm, followed by passage through both affinity columns used in the purification of the NADH and NADPH:FMN oxidoreductases.

Membrane Preparation. Membranes which contained NADH oxidase activity and which were used to prepare the solubilized dehydrogenase were obtained by a method similar to Kaback (1971) without the lysozyme/EDTA step. Cells from 600 mL of culture, grown to 3/4 log phase (2.2 OD_{660}) were harvested via centrifugation at 10 000*g* for 10 min and washed once in a buffer of 0.033 M potassium phosphate (pH 7.0) containing 3% NaCl. The cells were then resuspended in 10 mL of this buffer and poured into 1200 mL of a lower ionic strength buffer of 0.05 M potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$ and 5×10^{-4} M dithiothreitol. This treatment results in lysis of the cells. The suspension was brought to 25 °C and 10 μg/mL of DNase I was added. After 30 min, the lysate was centrifuged at 17 000*g* for 20 min and and the resulting pellet was resuspended by homogenization in the lysing buffer. A second incubation with DNase I at 40 μg/mL was performed. The membranes were then washed five times by homogenization in 0.1 M potassium phosphate (pH 7.0) with 10^{-4} M dithiothreitol.

Enzyme Assays. All spectrophotometric assays were carried out at 23 °C by monitoring the initial rate of oxidation of NADH or NADPH as the decrease in optical density at 340 nm using a Cary Model 14 recording spectrophotometer and cuvettes with a 1-cm pathlength.

An assay reaction is initiated by adding 0.1 mL of 2×10^{-3} M NADPH or NADH in 0.1 M buffer to 0.9 mL of 0.1 M buffer of the same pH containing the stated final concentration of acceptor and a sample of enzyme. Membrane-bound NADH dehydrogenase is assayed in the presence of 10 mM KCN to inhibit NADH oxidase activity. Unless otherwise stated, buffers at pH 5.6 and 7.0 are 0.1 M potassium phosphate and the buffer at pH 8.5 is 0.1 M potassium pyrophosphate. All values were corrected for the oxidation of NADH or NADPH that occurred in the absence of enzyme.

The NADH and NADPH:FMN oxidoreductases and the membrane bound and solubilized NADH dehydrogenase were also assayed in a coupled reaction with luciferase (Hastings et al., 1965) using an Aminco Chem-Glo photometer. The maximum initial intensity is measured upon the addition of 0.1 mL of 2×10^{-4} M NADH or NADPH, in 0.1 M potassium phosphate buffer (pH 7.0), into 0.5 mL of 0.1 M phosphate buffer (pH 7.0) containing 2.3×10^{-6} M FMN, 0.0005% v/v decanal, a fixed excess amount of purified luciferase and limiting oxidoreductase. Activity is thus expressed in arbitrary light units. Luciferase assays were performed by the injection of 0.1 mL of 1.5×10^{-4} M $FMNH_2$, photoreduced in the presence of 5 mM EDTA (Lee, 1972) into 0.5 mL of a solution containing luciferase, 0.0005% decanal, and 0.1 M phosphate buffer (pH 7.0). Initial light intensity was measured in an Aminco Chem-Glo photometer and recorded on an Aminco recorder. The initial peak light intensity is linear with respect to added luciferase.

Results

The Michaelis–Menten constants were determined for both oxidoreductases from initial velocity measurements (decrease in optical density at 340 nm) using various concentrations of one substrate under conditions of saturating concentration of the other substrate. These values are given in Table I. The K_ms were determined at the optimum pH of 8.5 and 5.6 for the NADH and NADPH:FMN oxidoreductases, respectively. The K_ms were also determined at pH 7.0. No significant differences were observed for the K_ms with respect to FMN. The K_ms for NADH and NADPH were fourfold and twofold higher for the NADH and NADPH:FMN oxidoreductases, respectively, at pH 7.0 (Jablonski & DeLuca, 1977).

The apparent K_ms for NADH, NADPH, and FMN were

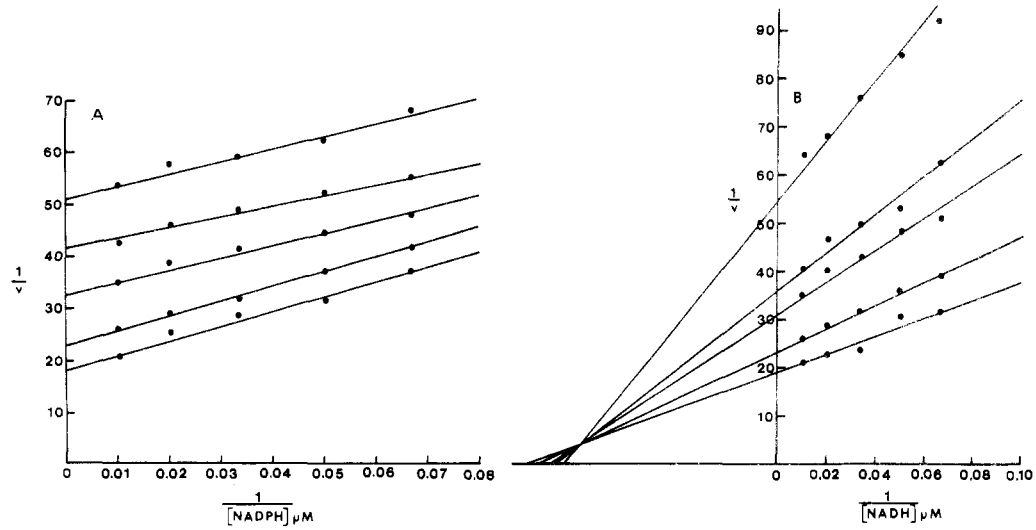

FIGURE 1: (A) Lineweaver-Burk plot of the activity of NADPH:FMN oxidoreductase at pH 5.6. Reciprocals of the initial reaction velocities in micromoles of NADPH oxidized per minute per milliliter of reaction mixture are plotted against the reciprocals of the NADPH concentrations at fixed FMN concentrations. FMN concentrations were 100 μM, 32 μM, 16 μM, 11 μM, and 7 μM, from bottom to top. (B) Lineweaver-Burk plot of the activity of NADH:FMN oxidoreductase at pH 8.5. Reciprocals of the initial reaction velocities in micromoles of NADH oxidized per minute per milliliter of reaction mixture are plotted against the reciprocals of the NADH concentrations at fixed FMN concentrations. Concentrations of FMN were 10 μM, 2 μM, 1 μM, 0.7 μM, and 0.4 μM, from bottom to top.

also determined in the coupled assay with luciferase, Table I, by measuring the maximum light intensity. A decrease for all K_ms was observed in the coupled assay as compared with the spectrophotometric assay. Duane & Hastings (1975) found a similar decrease in K_ms in the coupled reaction for the enzyme from *Photobacterium fischeri* and suggested this might be due to complex formation between the oxidoreductase and luciferase. An alternative explanation is that the luciferase because of its low turnover number is the rate-limiting reaction in the sequence and it is saturated with $FMNH_2$ at a NAD(P)H concentration where the oxidoreductase is not operating at maximal velocity. This would produce an apparent decrease in the K_m for NADH, NADPH, or FMN when one is measuring light as the product. If this is the explanation then by using a large excess of luciferase over oxidoreductase, conditions where the oxidoreductase is the limiting component should result in higher apparent K_ms. We have measured the K_ms with different ratios of oxidoreductase to luciferase and in all cases we find the same value for the K_ms in the coupled assay. The data are consistent with but do not prove the formation of a complex as suggested previously (Duane & Hastings, 1975). If a complex is formed both oxidoreductase and luciferase may be altered with respect to the binding sites for pyridine nucleotide and or flavin. Alternatively, the complex may protect $FMNH_2$ from autooxidation such that there is a greater efficiency of $FMNH_2$ utilization and light production by luciferase.

The apparent K_ms were also determined by observing the disappearance of NADH or NADPH by decrease in optical density at 340 nm in the presence of decanal and a large excess of luciferase. In this case the K_ms and the absolute activities of the oxidoreductases were unchanged from those determined in the absence of luciferase. The presence of luciferase does not appear to change the enzymatic characteristics of the oxidoreductases as observed spectrophotometrically.

Figures 1A and 1B are Lineweaver-Burk plots of the oxidoreductases at different fixed FMN concentrations. The NADPH:FMN oxidoreductase exhibits double displacement or ping-pong kinetics. The NADH:FMN oxidoreductase exhibits single displacement or Bi-Bi kinetics but the initial velocity measurements obtained do not discriminate between random or ordered mechanisms. From the data of Figure 1, the apparent order of the reactions with respect to all three substrates can be determined from Hill plots. In all cases, the order was 1.

Inhibition of the Coupled Reaction by FMN. The coupled reaction of either oxidoreductase with luciferase has a definite optimal FMN concentration. Increasing this FMN concentration above the optima, which is 2.3 μM and 21 μM for the NADH and NADPH specific coupled reactions, respectively, results in an inhibition of light output. Increased FMN does not inhibit the oxidoreductases when assayed spectrophotometrically. This inhibition cannot be explained as an absorption of emitted light by FMN since the effective optical densities at 490 nm for FMN at 2.3 μM and 21 μM are 9×10^{-3} and 8×10^{-2} units, respectively. A more likely explanation is that additional FMN does not result in an equal additional production of $FMNH_2$ but acts to increase autooxidation of enzyme associated $FMNH_2$. Free FMN can compete with luciferase for enzyme bound $FMNH_2$ leading to the formation of free $FMNH_2$ which is more susceptible to autooxidation. Various dyes and ferricyanide have been shown to inhibit light output in a coupled reaction indicating that other electron acceptors can function in a similar manner. Earlier evidence suggests that luciferase bound $FMNH_2$ is less susceptible to autooxidation (McElroy & Green, 1955).

pH Profile. The pH profile for the NADPH:FMN oxidoreductase is shown in Figure 2. The activities were determined at concentrations of 2.0×10^{-4} M NADPH, and 2.0×10^{-4} M FMN. These concentrations were found to be optimal at pH 7.0, 5.6, and 8.5. The enzyme exhibits a maximum activity at pH 5.5 which drops to a broad shoulder from pH 6.5 to pH 8.5 with an activity 75% that of maximum at pH 7.0. This profile is similar to that reported for the NAD(P)H:FMN oxidoreductase of *Photobacterium fischeri* (Duane & Hastings, 1975). The pH profile for the NADH:FMN oxidoreductase was also determined and found to be the same as obtained by Duane & Hastings (1975). This pH curve is bimodal, with maximal activity exhibited at pH 8.6, a minimum at pH 6.0, and a second maximum at pH 5.0.

Attempts to Measure Binding of the Oxidoreductases to Luciferase. If the oxidoreductases are capable of forming a complex with luciferase at a common binding site, it might be expected that one could observe a competition between the two oxidoreductases for luciferase. We looked for this by adding a large excess of NADH:FMN oxidoreductase to a coupled assay mixture containing NADPH:FMN oxidoreductase and luciferase and the reciprocal experiment. The addition of up to a 100-fold excess of one oxidoreductase over the other oxidoreductase and luciferase did not decrease the light intensity below that of a control value. This was true even if the potential competing oxidoreductase was added to luciferase prior to the addition of the other oxidoreductase and its reduced pyridine nucleotide. We observed a slight stimulation of light emission upon addition of excess NADH:FMN oxidoreductase to a coupled reaction of NADPH:FMN oxidoreductase and luciferase. This is due to the fact that the NADH enzyme will use NADPH as a substrate to a limited extent. The results suggest that, if a complex is formed, it is dependent on the enzyme bound $FMNH_2$ reacting with luciferase to give an oxidoreductase-$FMNH_2$-luciferase complex.

Appearance of NADPH:FMN Oxidoreductase during Cell Growth. Upon inoculation of a fresh culture of *Beneckea harveyi* into complete liquid media, logarithmic growth proceeds immediately. Luciferase synthesis lags for a period of time, then increases at a rate faster than growth. This process has been termed autoinduction and is dependent upon the production of an inducer by the bacteria (Nealson et al., 1970; Nealson, 1977). Luciferase is not synthesized until a sufficient concentration of the inducer has been produced in the media. NADH:FMN oxidoreductase production has been shown to parallel growth and is not autoinduced (Duane & Hastings, 1975; Meighen et al., 1976).

In order to determine if the NADPH:FMN oxidoreductase is also autoinduced, a large culture was started in a complete media and aliquots were removed as a function of time and assayed for growth (optical density at 660 nm), luciferase activity (in vitro light intensity), and oxidoreductase activity. The cells were quickly chilled and collected by centrifugation and lysed in 0.01 M phosphate buffer (pH 7.0) containing 1×10^{-3} M EDTA and 5×10^{-4} M dithiothreitol. This crude lysate was centrifuged to remove cell debris and then assayed for both oxidoreductases by disappearance of NAD(P)H as described in Materials and Methods. Both oxidoreductases were found to be synthesized at a rate comparable to growth, while luciferase, as measured by both in vivo and in vitro light, accumulates at a faster rate. These results demonstrated that the NADPH:FMN oxidoreductase is not autoinducible with luciferase. These experiments were confirmed independently using smaller cultures and assaying the oxidoreductases in the coupled assay with luciferase (see Materials and Methods).

Comparison of the Soluble Oxidoreductases with the Membrane-Derived NADH Dehydrogenase. Membrane Preparation and Solubilization of the NADH Dehydrogenase. In order to determine if the NADH or NADPH:FMN oxidoreductases were derived from the membrane-bound NADH oxidase, the activities of the particulate NADH oxidase and the NADH and NADPH:FMN oxidoreductases were assayed during the preparation and washing of the membrane fraction. Luciferase was also monitored as a control for soluble protein. The results are shown in Table II. Both NADH and NADPH:FMN oxidoreductases were washed from the membrane pellet along with luciferase, while the NADH oxidase remains with the pellet as expected. There is no evidence that either oxidoreductase is membrane bound. A small percentage of NADH oxidase activity is lost with each wash and

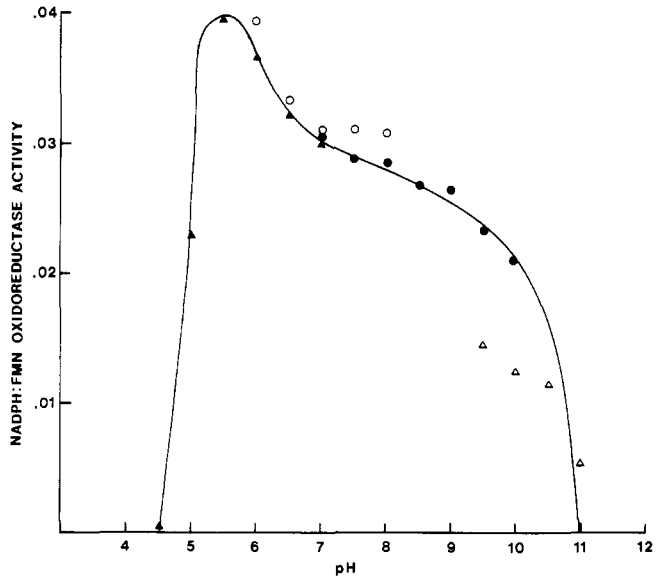

FIGURE 2: The effect of pH on the activity of NADPH:FMN oxidoreductase. Activity is expressed as micromoles of NADPH oxidized per minute per milliliter of reaction mixture. The buffers used were sodium pyrophosphate (●—●), potassium phosphate (○—○), sodium citrate-phosphate (▲—▲), and glycine NaOH (△—△). The final concentration of all buffers was 0.1 M.

is probably due to the disruption of the respiratory chain in some of the membrane fragments during homogenization and resuspension.

The membranes were solubilized by the addition of Triton X-100 to 1% v/v and incubated for 15 min at 25 °C followed by centrifugation to remove insoluble material. Virtually 100% of NADH oxidase activity is lost. The resulting solution contains a "solubilized" NADH dehydrogenase.

Light Production from the Bound and Solubilized NADH Dehydrogenase. The ability to produce light in a coupled reaction with luciferase was monitored before and after solubilization of the membrane bound NADH dehydrogenase. Due to the turbidity of the suspended membranes in the assay solution, the level of light intensity that could be generated with membrane-bound NADH dehydrogenase was very low. Even so, it was determined that solubilization increases light output 40–50% over that of membrane-bound dehydrogenase. This increase in activity is similar to the observed increase in activity by solubilization when artificial electron acceptors are employed and NADH oxidation is monitored by decrease in optical density at 340 nm.

The light production in the coupled assay by the solubilized NADH dehydrogenase from membranes was examined and compared with the light-producing ability of the NADH:FMN oxidoreductase. Both enzyme concentrations were adjusted such that equal volumes gave equal activities at pH 7.0 with 2.6×10^{-4} M FMN when assayed spectrophotometrically at 340 nm. The activity of these enzymes was also observed by following the reduction of FMN anaerobically by the decrease in optical density at 442 nm in a Thunberg tube, which confirmed that FMN was the acceptor and was being reduced enzymatically at the expense of NADH. Light production with luciferase was measured for each enzyme at 2.6×10^{-4} M FMN, 0.0005% decanal, and 0.02% Triton X-100 in 0.1 M potassium phosphate buffer (pH 7.0). FMN at this concentration is highly inhibitory of light output in the coupled assay and Triton X-100 was found to be inhibitory to the activity of luciferase. These less than optimal conditions for the coupled reaction were necessary to ensure the equal rate of utilization

TABLE II: Distribution of Luciferase, Oxidoreductases, and NADH Oxidase during Membrane Preparation from *Beneckea harveyi*.[a]

Fraction	Total luciferase (light units)	% recovery	Total NADH oxidase (μmol of NADH/min)	% recovery	Total NADH:FMN oxidoreductase (μmol of NADH/min)	% recovery	Total NADPH:FMN oxidoreductase (μmol of NADPH/min)	% recovery
Crude Lysate	209	100	135	100	32	100	31	100
Supernatant I	167	80	12	9	22	69	22	71
Pellet I	48	23	129	95				
Supernatant II	19	9	0	<1	2.7	8	3.1	10
Pellet II	35	16	122	90				
Supernatant III	28	13	0	<1	3.7	12	1.6	6
Pellet III	2	1	123	91				
Supernatant IV	2	1	0	<1	0	<1	0	<1
Pellet IV	0.1	0.04	116	86				
Supernatant V	0.06	0.02	0	<1	0	<1	0	<1
Pellet V	0.01	0.006	113	83				

[a] NADH:FMN oxidoreductase and NADH oxidase were assayed at pH 8.5. NADPH:FMN oxidoreductase was assayed at pH 5.6.

TABLE III: The Activity of Various Electron Acceptors as Substrates for the Oxidoreductases and NADH Dehydrogenase.[a]

Acceptor	Concn (M)	Rel % act. NADH:FMN oxidoreductase	Rel % act. NADPH:FMN oxidoreductase	Rel % act. NADH dehydrogenase: Membrane bound	Rel % act. NADH dehydrogenase: Solubilized
FMN	1.3×10^{-4}	100	100	54	54
	6.5×10^{-4}	100	100	100	100
O_2	2.0×10^{-4} [b]	0	0	900	0
DCIP	7.0×10^{-5}	13	135	1900	2500
Menadione	7.0×10^{-5}	6	100	1360	2320
$KFeCN_6$	5.0×10^{-4}	15	164	1680	2730
FAD	4.0×10^{-5}	75	58	45	27
	2.0×10^{-4}	50	100		54
Methylene blue	2.5×10^{-4}	3	23	2040	3640
DTNB	1.25×10^{-4}	4	85	0	0

[a] All activities are normalized to FMN as acceptor. The activity of the membrane bound dehydrogenase with acceptors other than oxygen was obtained in the presence of 10^{-2} M KCN. Both KCN and solubilization inhibit activity with oxygen 100%. The only difference between the membrane bound NADH dehydrogenase and the solubilized form is the presence of 1% v/v of Triton X-100 and the relative activities, therefore, are comparable. [b] O_2 concentration for a saturated aqueous solution at 25 °C (Chappell, 1964).

of NADH by both enzymes and the intensity of light generated here was readily detectable. The results showed that the solubilized NADH dehydrogenase could only generate light at 20% the intensity of the NADH:FMN oxidoreductase. Decanal was not inhibitory to the activity of the dehydrogenase at the concentration of the coupled reaction.

Acceptors of NADH and NADPH:FMN Oxidoreductases. Table III shows the relative activities obtained at saturating concentrations of a variety of electron acceptors and NADH or NADPH for both FMN oxidoreductases at their respective pH optima. FMN is the most efficient acceptor for the NADH specific enzyme with NADH as the donor. FAD is the only other acceptor utilized to an appreciable degree and becomes inhibitory above 4.0×10^{-5} M. All the acceptors were also examined in the presence of a catalytic amount of FAD, with no increase in activity observed. NADPH will serve as a substrate for this enzyme; however, the maximal velocity is only 15% of that obtained with NADH, and only FMN or FAD are active as acceptors.

In contrast, the NADPH:FMN oxidoreductase exhibits appreciable activity with all the acceptors except methylene blue. Several of these compounds are better substrates than FMN. FAD is not inhibitory above 4.0×10^{-5} M, and in fact the enzyme requires a concentration of 2.0×10^{-4} m for maximal activity with FAD. FAD does not alter the reaction rates of either enzyme when added with the other acceptors.

Acceptors of Membrane-Bound and Solubilized NADH Dehydrogenase. The various acceptors shown in Table III were tested with membrane bound and solubilized NADH dehydrogenase for their ability to oxidize NADH and NADPH. The concentrations of the electron acceptors and NADH were saturating. The membrane-bound dehydrogenase was assayed in the presence of 10 mM KCN which effectively inhibited NADH oxidase activity. NADPH could not be oxidized enzymatically with any of the acceptors available at any pH and transhydrogenase activity was not detectable in these preparations.

Both the membrane bound and solubilized NADH dehydrogenase exhibit high activity with DCIP, $KFeCN_6$, methylene blue, and menadione as acceptors, and very little activity with FMN and FAD as acceptors. This activity with FMN and FAD can be increased by utilizing a higher concentration of flavin, indicating a much higher K_m for flavin than that observed for the soluble oxidoreductases. Solubilization appears

TABLE IV: The Effect of Inhibitors on the Oxidoreductases, the Membrane NADH Oxidase, and the Solubilized NADH Dehydrogenase.[a]

Inhibitors	Concn (M)	% inhibition			
		NADH:FMN oxidoreductase	NADPH:FMN oxidoreductase	NADH oxidase	NADH dehydrogenase solubilized
AMP	1.0×10^{-2}	36	28	0	10
Dicoumarol	3.25×10^{-4}	0	15	35	40
o-Phenanthroline	5.0×10^{-3}	52	14[b]	20	24
KCN	1.0×10^{-2}	0	10	100	0
DNP	1.4×10^{-4}	0	0	0	5
HQNO	1.0×10^{-4}	0	0	100	0
Rotenone	4.0×10^{-4}	20	30	23	12
DTNB	1.0×10^{-3}	38			45
NEM	1.0×10^{-3}	38	26		7
PMB	1.0×10^{-3}	49	80		100
Triton X-100	1%	0	0	100	0

[a] DTNB, NEM, and PMB were incubated with enzyme at pH 8.0 for 30 min at 4 °C. Samples were then assayed for activity at the optimum pH of the enzymes. All other inhibitors were tested without incubation at the optimum pH of the enzymes. [b] At pH 7.0 inhibition was 35%.

to increase the activity of the NADH dehydrogenase with all the acceptors. Assays with NADH dehydrogenase were typically done at pH 8.5; if pH 7.0 and pH 5.6 buffers were used, the activity was depressed.

The Effect of Inhibitors on the Soluble and Membrane-Derived Enzymes. Table IV shows the effect of inhibitors on the soluble oxidoreductases, the NADH oxidase and the NADH dehydrogenase which has been solubilized from the membrane by Triton X-100. AMP up to 10 mM only partially inhibits both the soluble enzymes and has little effect on the membrane enzymes. KCN, HQNO, rotenone, and dicoumarol as expected all inhibit the NADH oxidase significantly. Rotenone also inhibits to a degree both soluble oxidoreductases, but this is probably a nonspecific inhibition, unrelated to the classical rotenone block. *o*-Phenanthroline, an iron chelator, significantly inhibits the NADH:FMN oxidoreductase and also the NADPH:FMN oxidoreductase at pH 7.0. All of the enzymes are sensitive to sulfhydryl reagents, although the NADH:FMN oxidoreductase seems to be most resistant. Both Mg–ATP and cAMP at 2.6×10^{-4} and 2.0×10^{-4} M, respectively, were tested for any effects upon the oxidoreductases and the Triton X-100 solubilized NADH dehydrogenase. No changes in activity were observed.

Discussion

The presence of soluble NAD(P)H:FMN oxidoreductases which are capable of supplying $FMNH_2$ to luciferase for light production constitutes an electron transport system which can presumably compete with the cytochromes for reduced pyridine nucleotides. The interaction between the luminescent system and electron transport via the cytochromes has been demonstrated by the fact that at certain stages of cell growth luminescence can be stimulated by the addition of cyanide (Van Schouwenberg, 1938; Nealson et al., 1970).

It would seem that there should be some cellular control which prevents NADH and NADPH from being depleted through autooxidizable $FMNH_2$ and light production rather than through the cytochrome system.

The results presented here still do not answer unequivocally the question of the role of the NADH and NADPH:FMN oxidoreductases in controlling in vivo bioluminescence. The NADPH:FMN oxidoreductase was shown to be constitutive and therefore neither of the oxidoreductases are co-induced with luciferase. Both of the oxidoreductases, as well as luciferase, appear to be completely soluble in these bacteria unlike *Photobacterium leiognathi,* where it has been reported that part of the luciferase is a glycoprotein which is membrane bound (Balakrishnan & Langerman, 1977).

With regard to complex formation of the oxidoreductases with luciferase, the K_ms for NADH, NADPH, and FMN are significantly decreased in the coupled assay when compared with the spectrophotometric assay of the oxidoreductases alone. This is consistent with complex formation. Also, it appears that the soluble NADH:FMN oxidoreductase is more efficient in light production in the coupled assay than a comparable amount of the NADH dehydrogenase which was obtained from the membranes. This is also consistent with the formation of a complex of the soluble enzyme with luciferase such that there is an efficient transfer of $FMNH_2$ from oxidoreductase to luciferase.

If such a complex is formed, there is no apparent competition between the two oxidoreductases for luciferase since a large excess of one oxidoreductase had no apparent effect on the coupled assay with the other enzyme. The presence of luciferase does not affect the ability of the oxidoreductases to oxidize NADH or NADPH. If a complex need exist for light production, it is difficult to see how it alone prevents depletion of NAD(P)H levels in the cell.

The kinetic studies on the soluble oxidoreductases revealed an interesting difference between the two enzymes, namely, that the NADH:FMN oxidoreductase enzyme exhibits Bi–Bi kinetics while the NADPH:FMN oxidoreductase exhibits ping-pong type kinetics. The simplest explanation is that this enzyme contains a bound flavin, Fe and/or both, which is reduced by NADPH and this reduced enzyme then reacts with FMN.

The comparison between the soluble oxidoreductases and the NADH dehydrogenase obtained from the membrane with various acceptors and inhibitors clearly differentiates between these enzymes. The most striking difference is that both soluble NADH and NADPH:FMN oxidoreductases will use FMN or FAD as substrates while the membrane bound or Triton X-100 solubilized dehydrogenase uses FMN or FAD to only a very limited extent (Table III). The soluble NADH:FMN oxidoreductase is in fact quite specific for FMN or FAD while the NADPH:FMN enzyme will use a variety of different electron acceptors. The lack of significant inhibition by 10 mM AMP distinguishes these enzymes from the *E. coli* NADH dehydrogenase studied by Dancy et al. (1976). Der Vartanian (1976) has isolated a soluble dehydrogenase from *Azotobacter vinelandii,* and this enzyme contains FMN and some iron-

sulfur centers. It will also use NADPH as a substrate, however, with a lower V_{max}.

Puget & Michelson (1972) demonstrated the presence of soluble NADH:FMN oxidoreductases from several different bacteria; however, they have not been purified or well characterized. The role of these enzymes is unknown at present. Studies are under way to further characterize these interesting enzymes.

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Glycoproteins of the CHO Cell Membrane: Partial Fractionation of the Receptors for Concanavalin A and Wheat Germ Agglutinin Using a Lectin Immunoprecipitation Technique†

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ABSTRACT: The lectin-binding glycoproteins of the CHO cell plasma membrane have been analyzed using an immunoprecipitation technique. Membranes from cells labeled via metabolic incorporation of [^{3}H]glucosamine were solubilized in deoxycholate, the solubilized material was treated with concanavalin A, *Ricinus communis* agglutinins, or wheat germ agglutinin, and the lectin–glycoprotein complexes were precipitated with specific antisera directed against the lectins. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and fluorescence-enhanced autoradiography. The CHO cell membrane contained two major glucosamine-labeled glycoprotein classes, A and B, which migrated as diffuse bands on polyacrylamide gels, and which had apparent molecular weights of 100 000 and 130 000, respectively. Several minor labeled components were also apparent. Lectin immunoprecipitation of solubilized CHO cell membranes with wheat germ agglutinin resulted in the precipitation of material primarily of class A, while immunoprecipitation with concanavalin A produced material of class B. Thus a degree of subfractionation of the membrane glycoproteins according to lectin-binding specificity has been obtained. The utility of the lectin immunoprecipitation technique is discussed in terms of analyzing the molecular associations between subclasses of membrane glycoproteins and nonglycosylated membrane macromolecules.

The biochemical nature, surface distribution, and lateral mobility of cell surface glycoproteins seem to be directly related to important biological processes such as the expression of malignant transformation, cell to cell recognition, and control of cell growth (Nicolson, 1974). Since glycoproteins have an affinity for lectins, the carbohydrate binding proteins derived from plant sources, surface glycoproteins frequently have been termed lectin "receptors". In the past few years a large number of investigations have dealt with the biological effects of lectin binding (Burger, 1973), and with the relationship between the mobility and distribution of lectin receptors and the actions of the cytoskeleton (Nicolson, 1976). Most of the studies im-

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