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Identification of NADH-Specific and NADPH-Specific FMN Reductases in *Beneckea harveyi*

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Distinct FMN reductases specific for NADH and NADPH were identified in extracts of *Beneckea harveyi*. These enzymes differ in their physical (molecular weight, thermostability) as well as in their chemical properties (binding constants for NADH and NADPH). The NADH-specific enzyme is more efficient than the NADPH-specific one with respect to the bioluminescent reaction.

Some years ago Strehler and Cormier [1] and McElroy *et al.* [2] identified the components required for maximum luminescence in a cell-free extract of *Photobacterium fischeri*, *i.e.* $FMNH_2$, a long-chain aldehyde, oxygen and luciferase. $FMNH_2$ which readily oxidises in the presence of O_2 is generated by the oxidation of NADH or NADPH through an enzyme which has been given the names of FMN reductase or NAD(P)H dehydrogenase [3]. In wild-type *Photobacterium fischeri*, it is difficult to separate the dehydrogenase activity from luciferase, suggesting a possible association *in vivo* between the two activities [4]. Using a purification procedure adapted from the method of Gunsalus *et al.* [5], we were able to separate and characterize NADH-specific and NADPH-specific FMN reductase activities in *Beneckea harveyi*.

MATERIALS AND METHODS

Growth and Harvesting of Cells

Beneckea harveyi cells (previously described as *Photobacterium fischeri* strain MAV [6] and recently identified by Reichelt and Baumann [7]), kindly provided by Dr J. W. Hastings, were subcultured in the solid agar medium previously described [5]. The cells were grown at 25 °C with vigorous aeration; anti-foam was added to the medium. Cells were cooled at the time of maximum luminescence, at a density of 1×10^9 cells/ml, and harvested by continuous centrifugation in a Sorvall centrifuge (rotor SS 34) at a speed of 16000 rev./min and a flow rate of 250 ml/min. The yield of packed wet cells was about 4 g/l medium.

Purification Procedure

We followed the method of Gunsalus-Miguel *et al.* [5], with minor modifications for the purification of luciferase. Frozen cells were thawed and lysed in cold water adjusted to pH 7.0 with NaOH and containing 0.1 mM dithioerythritol, 5 mM $MgSO_4$ and a trace of DNase, in a ratio of 6 ml/g wet pellet. After overnight stirring at 4 °C, EDTA was added to a final concentration of 15 mM. The crude extract was centrifuged for 30 min at 8000 rev./min. Dry DEAE-cellulose (Whatman DE-32) was added to the supernatant (0.22 g/g wet pellet); the pH was maintained at 7.0 by the addition of 0.5 N acetic acid. Protein was extracted batchwise by increasing phosphate concentrations (0.1, 0.15, 0.5 M potassium phosphate pH 7.0). The fraction extracted by 0.5 M phosphate was then fractionated with ammonium sulfate (special enzyme grade, Mann Research Laboratories). Material precipitating between 40 and 75% saturation was dissolved in 0.25 M phosphate buffer pH 7.0 (0.1 mM dithioerythritol) and dialysed for 16 h against a large volume of the same buffer. The sample was then chromatographed on DEAE-Sephadex A50 (Pharmacia). The gel column (2.4×100 cm) equilibrated with 0.25 M phosphate buffer pH 7.0, was eluted with 3 l of the same buffer, followed by 2 l of 0.35 M buffer. The active fractions were pooled, precipitated with ammonium sulfate between 40 and 75% saturation and dialysed against 0.1 M phosphate buffer pH 7.0 (with

Abbreviations. $\bar{K}$, dissociation constant of the binary EA complex, $\bar{K}_A$ = [E] [A]/[EA]; K, dissociation of the ternary EAB complex, K_A = [EB] [A]/[EAB].

Enzymes. Luciferase; NADH-specific and NADPH-specific FMN reductase (EC 1.6.99.–); alcohol dehydrogenase (EC 1.1.1.1).

1 mM EDTA and 0.1 mM dithioerythritol). The concentrated active material (respectively NADH and NADPH-specific FMN reductase activities and luciferase) obtained by DEAE-Sephadex chromatography, was then separately applied on a Sephadex G-100 column (2.4 × 100 cm) equilibrated with 0.1 M phosphate buffer pH 7.0 and eluted with the same buffer. The final reductase preparations were estimated to be less than 20% pure, based on the Sephadex chromatogram and on polyacrylamide gel electrophoresis [8]. The active fractions were pooled, concentrated by ultrafiltration and kept frozen at −20 °C. All experiments were done with these preparations.

Protein Determination

Protein was determined according to Lowry *et al.* [9], with bovine serum albumin (Sigma Chemical Co.) as a standard.

Enzyme Assays

Luciferase. Luciferase activities were measured using reduction by dithionite: 2 ml FMN solution (0.2 mM in 0.02 M phosphate buffer pH 6.8), 10 µl enzyme solution and 1 µl decylaldehyde were rapidly injected into a reaction vial containing 2 mg freshly weighed dithionite. The vial was shaken for a few seconds and the bioluminescence was measured with a Packard 2002 scintillation counter, the coincidence circuit of which was switched off. The counter was operated in the repeated mode over periods of 0.1 min. The light output rapidly decreased, but reached a steady state after about 4 min. At this excess dithionite concentration, FMN was found to remain reduced (as judged by the lack of absorbance at 445 nm) for more than 3 h, indicating that the solution was depleted of oxygen. The steady level of bioluminescence derives from small quantities of oxygen diffusing in, since the luciferase · $FMNH_2$ complex can very effectively compete with the excess dithionite for the O_2 molecules [10]. The light intensity is proportional to the luciferase concentration over the whole time interval, but better reproducibility was found at the steady level.

NADH-Specific and NADPH-Specific FMN Reductase. The FMN-dependent dehydrogenase activities were assayed at 23 °C by following the oxidation of NADH or NADPH spectrophotometrically at 340 nm, using a Cary Model 118 recording spectrophotometer. The reaction mixtures contained FMN (130 µM, calculated on its absorbance at 445 nm with an absorption coefficient of 12200 M^{-1} cm^{-1}), NADH or NADPH (160 µM, calculated on its absorbance at 340 nm with an absorption coefficient of 6220 M^{-1} cm^{-1}) and limiting amounts of enzyme in a total volume of 3 ml 0.02 M phosphate buffer pH 6.8.

Coupled Assay. As luciferase utilizes $FMNH_2$, the FMN reductases can be detected by means of the luciferase reaction. Their efficiencies in the bioluminescent reaction were compared according to this principle. The reaction mixtures contained FMN (2.5 µM), NADH or NADPH (1.6 µM), luciferase (0.02 µg/ml), 20 µl of a saturated aqueous aldehyde solution and different amounts of NADH-specific of NADPH-specific FMN reductase in a total volume of 3.12 ml 0.02 M phosphate buffer pH 6.8. Bovine serum albumin was added (0.1 mg) to stabilize the luciferase [11]. 400 µl of the reaction mixture was used for measuring the luminescence, the rest for following NADH or NADPH oxidation. The same kind of assay was used for determination of some of the dissociation constants for FMN, NADH and NADPH. For these experiments 0.1 µg NADH-specific reductase or 0.7 µg NADPH-specific reductase were used in a 313 µl volume. Other concentrations (except for NADH or NADPH and FMN) were as mentioned above.

Alcohol Dehydrogenase. Activity of yeast alcohol dehydrogenase was determined following the method of Vallee and Hoch [12].

Sucrose-Gradient Centrifugation

This was carried out according to Martin and Ames [13]. Linear 5–20% sucrose gradients (11 ml), buffered with 0.02 M phosphate pH 6.8, plus 0.1 mM dithioerythritol, were prepared with an Isco gradient former. About 50 µg (in a volume of 100 µl) of the enzyme preparations were loaded on top of the gradient. After centrifugation in a L5-65 Beckman ultracentrifuge during 26 h at 4 °C and 40000 rev./min with a SW41 rotor, 250-µl fractions were collected and assayed for enzymic activity. The distance (d) travelled by a protein in such a gradient can be correlated to its molecular weight by the formula

$$\frac{d_1}{d_2} = \left(\frac{M_{r1}}{M_{r2}}\right)^{2/3}.$$

Thermostability

Small amounts, about 0.5 µg, of the NADH-specific or NADPH-specific FMN reductase preparation, were incubated at different temperatures in 0.1 ml 0.1 M phosphate buffer pH 6.8, 0.1 mM dithioerythritol, 1 mM EDTA. After 5-min incubation the samples were diluted in ice-cold 0.02 M phosphate buffer pH 6.8 and the residual enzymic activity was measured spectrophotometrically or by the coupled assay at 23 °C.

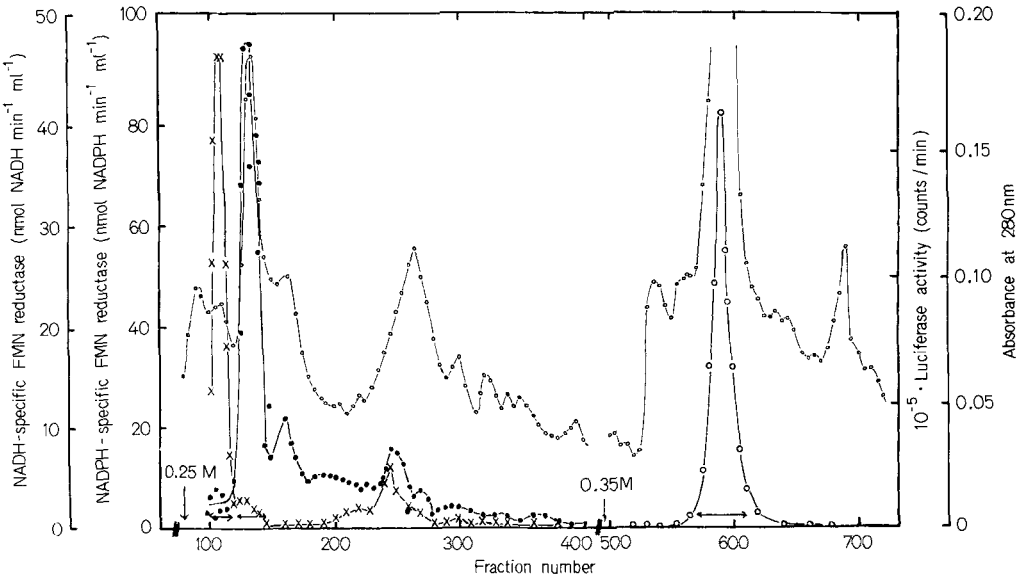

Fig. 1. *DEAE-Sephadex column chromatography of a partially purified* B. harveyi *extract.* The fraction collected between 40–75% ammonium sulfate saturation was dialyzed against 0.25 M phosphate buffer, 0.1 mM dithioerythritol at pH 7.0, and applied onto a 2.4 × 100-cm column equilibrated with the same buffer. The fractions (5.9 ml) were assayed for absorbance 280 nm (o——o), for luciferase (O····O) and for FMN reductase activity with NADH (●----●) and NADPH (×——×) (as described in Methods and Materials)

Chemicals and Reagents

FMN (Sigma Chemical Co.), was purified by DEAE-cellulose chromatography following the method of Massey and Swoboda [14]. NADH (grade I), NADPH (grade I) and alcohol dehydrogenase were obtained from Boehringer. *N*-Decylaldehyde (Koch-Light Laboratories) was used after purification by vacuum distillation. In some experiments the undiluted material was used, in others a water-saturated solution. Bovine serum albumin and cytochrome *c* from horse heart were obtained from Sigma Chemical Co., dithioerythritol from Calbiochem.

Potassium phosphate buffers were used throughout. All other reagents were of analytical grade when available.

RESULTS AND DISCUSSION

The greatest part of the NADH and NADPH dehydrogenase activities are separated from the luciferase activity after treatment of the crude extract with DEAE-cellulose. The activity which remains associated with the luciferase after DEAE-cellulose adsorption and ammonium sulphate fractionation is separated from it by chromatography on DEAE-Sephadex. At the same time the NADH-specific and NADPH-specific FMN reductase activities appear as two well-resolved peaks (Fig. 1), suggesting that different enzymes are involved.

Table 1 summarizes the purification procedure. The yield in FMN reductases is very low since we were actually interested in the purification of luciferase, and recovered only that activity that remained associated with luciferase up to the DEAE-Sephadex chromatography step. Furthermore it should be mentioned that our reductase preparations are subject to considerable loss of activity upon standing at 4 °C and even at −20 °C.

Puget and Michelson [15] reported that FMN reductase isolated from *Photobacterium sepia* also suffered considerable loss of activity upon standing. Their enzyme preparation could be stabilized in the presence of 20% glycerol. However this was not verified with our preparations.

The purification factor, calculated on the basis of the enzymic activity per mg protein, was 90 for the NADH-specific FMN reductase, 140 for the NADPH-specific enzyme while luciferase was purified about 50-fold.

The molecular weights of the several enzymes were estimated by gel filtration on Sephadex G-100, with reference to cytochrome *c* (M_r 12400), serum albumin (M_r 67000) and yeast alcohol dehydrogenase (M_r 151000). The NADH-specific enzyme was assigned a molecular weight of 19000 and the NADPH-specific reductase one of 40000. The molecular weights as estimated by linear sucrose-gradient centrifugation using yeast alcohol dehydrogenase as reference were about 1.6 times larger (31000 and 63000). Our values obtained for the NADH-specific FMN reductase agree quite well with those reported by Puget and Michelson [15] for their NADH : flavin oxidoreductase (23500) and Duane and Hastings [3] for their

Table 1. *Purification of luciferase and separation of NADH-specific and NADPH-specific FMN reductases*
NADH and NADPH reductase activities were measured spectrophotometrically at 340 nm; luciferase activity was measured using the dithionite assay

Fraction	Volume	Total protein	NADH reductase		NADPH reductase		Luciferase
			specific activity	yield	specific activity	yield	yield
	ml	mg	nmol NADH min^{-1} mg^{-1}	%	nmol NADPH min^{-1} mg^{-1}	%	%
1. Crude extract	380	4180	19	100	11	100	100
2. DEAE-cellulose							
– supernatant	350	2340	30	100	6	33	12
– 0.5 eluate	165	500	26	16	23	25	70
3. $(NH_4)_2SO_4$ 40–75% saturation fraction	12	300	30	11	20	13	67
4. Concentrated preparations after DEAE-Sephadex and Sephadex G-100							
– NADPH-specific	1.2	1	–	–	1850	3	$<10^{-3}$
– NADH-specific	3.5	1	2050	2	–	–	$<10^{-3}$
– luciferase	3.6	28	–	<0.02	–	<0.02	34

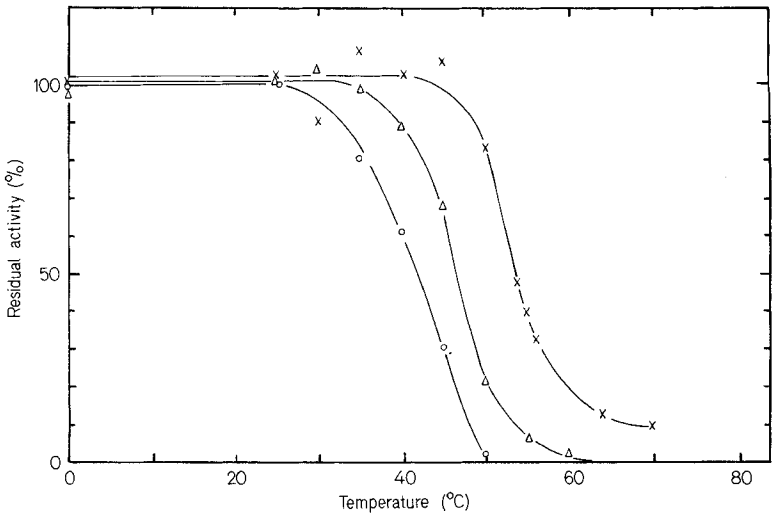

Fig. 2. *Thermostability of NADH-specific and NADPH-specific FMN reductase and of luciferase.* The percentage activity remaining after 5-min incubation at various temperatures for luciferase (O——O), NADPH-specific (Δ——Δ) and NADH-specific FMN reductase (×– – –×) (measured as described in Methods and Materials) is plotted against temperature

preparation referred to as NAD(P)H oxidoreductase (24000).

In order to further differentiate the two reductases we studied their thermostability. Fig. 2 shows the percentage activity remaining after 5-min incubation at different temperatures: at 55 °C there is still 40% residual NADH-specific activity whereas the NADPH-specific reductase activity is completely abolished under the same experimental conditions. The denaturation process is strictly first order, *i.e.* the logarithm of residual activity *versus* time plot is linear at constant temperature.

Luciferase is not as heat-stable as either FMN reductase (see Fig. 2). We thus attempted to demonstrate a stabilization of luciferase by one of the reductase, which might indicate a degree of association between these enzymes. The results of these experiments were not conclusive. Attempts to demonstrate the existence of a complex between either one of the reductases and luciferase by centrifugation in sucrose gradients, in the presence or in the absence of the substrates, were also negative. This does not exclude however that such an association might exist under conditions *in vivo*. These results are in agreement

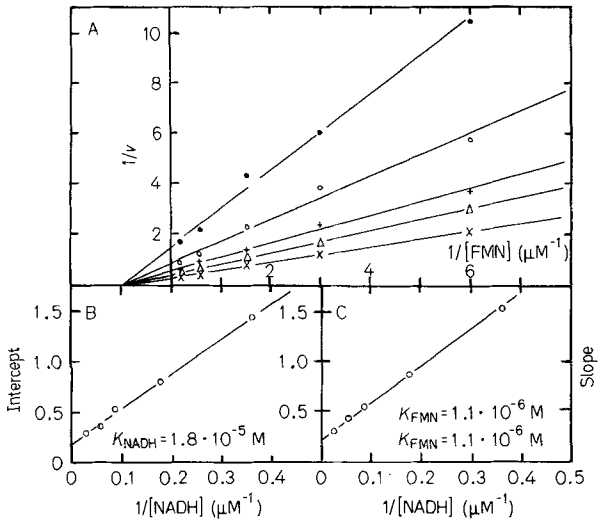

Fig. 3. *Graphical determination of the dissociation constants of the NADH-specific FMN reductase for NADH and FMN by the double-reciprocal plot.* (A) Primary plot: dependence of the reciprocal of the initial reaction rate ($1/v$) on the inverse of the FMN concentration at different NADH concentrations (2.8 μM, 5.5 μM, 11.4 μM, 17.2 μM, and 34.8 μM). (B) Secondary plot: intercept of the primary plot *versus* the reciprocal of the NADH concentration. (C) Secondary plot: slope of the primary plot *versus* the reciprocal of the NADH concentration. The reaction rate v was expressed in 10^{-2} $A_{340\,nm}$ units consumed in 5 min

with Puget and Michelson [15], who also failed to detect a reductase · luciferase complex in *Photobacterium fischeri* and other luminescent bacteria. Gibson *et al.* [16] however were able to demonstrate protection of *Ph. fischeri* luciferase against thermal denaturation in the presence of large reductase quantities.

The different dissociation constants were determined by spectrophotometrically measuring the initial reaction rates of NADH and NADPH oxidation at varying substrate concentrations, or by measuring the initial light intensity in a coupled reaction. Two different plotting procedures were used to determine the constants: the double-reciprocal plot of Linewaver-Burk [17] as in Fig. 3, and the direct linear plot of Eisenthal and Cornish-Bowden [18] as in Fig. 4. The values obtained by both methods agree quite well. The values for the dissociation constants of the different binary and ternary complexes ($\bar{K}$ and K respectively) for both enzymes listed in Table 2, are the means of the data obtained by the different measuring and plotting methods.

Good agreement was found between the spectrophotometric and luminescence measurements in the

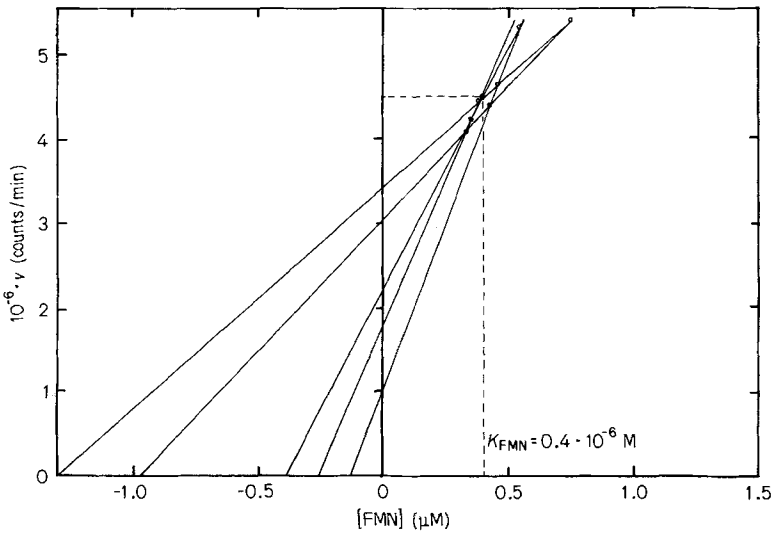

Fig. 4. *Graphical determination of the dissociation constant of the NADPH-specific FMN reductase for FMN by the direct linear plot [18].* For each measurement a line is drawn through the value of the initial reaction rate on the y axis (measured by the coupled assay as described in Methods and Materials) and the FMN concentration set on the $-x$ axis. The median intersection point of the different lines provides the value of K_{FMN}. The NADPH concentration was 3 μM

Table 2. *Dissociation constants of the different binary ($\bar{K}$) and ternary (K) complexes of the NADH-specific and NADPH-specific FMN reductases from* Beneckea harveyi
n.d. = not determined

FMN reductase	$10^6 \times$ Dissociation constant					
	K_{FMN}	$\bar{K}_{FMN}$	K_{NADH}	$\bar{K}_{NADH}$	K_{NADPH}	$\bar{K}_{NADPH}$
	M					
NADH-specific	1.1	1.1	17	17	>1000	n.d.
NADPH-specific	0.5	0.3	>1000	n.d.	0.5	0.3

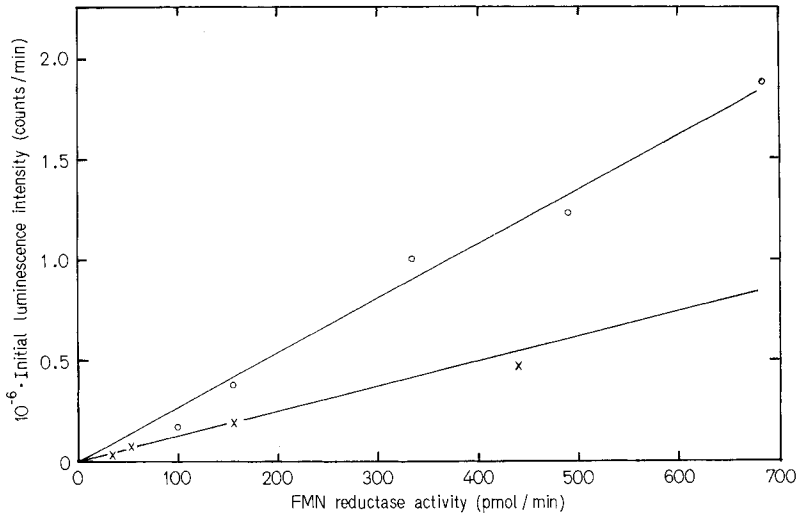

Fig. 5. *Efficiency of the NADH-specific and NADPH-specific FMN reductases in the bioluminescent reaction as measured by the coupled assay.* The initial luminescence intensity is plotted against the NADH (O ——O) or NADPH (× ——×) dehydrogenase activity (expressed as pmol substrate oxidized per min in the reaction volume)

determination of the constants for the binding of FMN and NADH to NADH-specific FMN reductase. An advantage of the coupled assay is that it is far more economical than the spectrophotometric assay: about 10 to 100 times less enzyme is needed per assay. This is a consequence of the high sensitivity obtained in light measurements with a scintillation counter. Another advantage of the method is its great dynamic range. The spectrophotometer cannot be used for measuring the reaction rates with the NADPH-specific reductase, since the NADPH concentrations used (about 1 μM) were too low to be measured at 340 nm. The reactions of the NADPH-specific reductase with NADH and of the NADH-specific reductase with NADPH could not be measured spectrophotometrically either: the concentrations used here (of the order of 1 mM) have too high absorbance at 340 nm. These measurements were made by the coupled assay, at a constant high FMN concentration (3 μM). The values of these dissociation constants are only approximate: a large error results from the non-enzymic reaction, which proceeds at an appreciable rate (up to 100% of the enzymic rate) at these high nucleotide concentrations. The NADH-specific and the NADPH-specific FMN reductases seem to be active with both nucleotides as substrates. The affinity of the NADH-specific reductase is about 60 times greater for NADH than for NADPH, while the affinity of the NADPH-specific reductase for NADPH is 1000 times greater than for NADH. No NADH was detected in the NADPH sample and *vice versa* by paper chromatography [19] which allowed us to detect two parts per thousand.

The fact that $\bar{K}$ is equal or nearly equal to K indicates that the enzymes obey a random mechanism, *i.e.* the binding of one substrate (*e.g.* FMN) does not affect the binding of the other (NADH or NADPH) to an appreciable extent.

The affinities of both enzymes for FMN are similar but differ significantly (the K_m for the NADH-specific reductase being 1.1 μM while, the NADPH-specific enzyme has a K_m of 0.5 μM). The determination of the primary structure of these enzymes may prove to be very interesting for the study of the interaction between small molecules and proteins.

Fig. 5 shows the initial light intensity at constant luciferase concentration as a function of the dehydrogenase activity for the two enzymes. It can be seen that for a same dehydrogenation rate, measured at 340 nm, the initial light intensity obtained with the NADH-specific reductase is about 2.4 times that obtained with the NADPH-specific enzyme. The higher efficiency of the NADH-specific enzyme in the bioluminescence reaction suggests a closer association to luciferase.

The molecular weight and pH profile (not shown) of our NADH-specific FMN reductase preparation are similar to those of the FMN reductase of *Beneckea harveyi* studied by Duane and Hastings [3]. The specific activities of our NADH-specific and NADPH-specific reductases are about 2 μmol NADH (respectively NADPH) oxidized per mg protein per min, compared to 0.24 μmol min^{-1} mg $protein^{-1}$ reported by Duane and Hastings [3]. This, together with the appreciable activity with NADPH of their preparation, led us to suppose that their preparation contained some of the NADPH-specific enzyme. It would be very interesting to verify if in the preparations from *Photobacterium fischeri*, M_r 43000 [3] and *Photobacterium sepia*, M_r 23500 [15] two FMN reductases, a NADH-specific and a NADPH-specific one, could be resolved.

Over the past few years, the bacterial luciferase system has found application in the assay of numerous substances [20]. The use of purified FMN reductase would greatly enhance the specificity of the method with regard to the nicotinamide adenine nucleotides. When using unpurified dehydrogenase preparations, NADH interferes in the assay of NADPH (and *vice versa*). Thus samples to be assayed must be treated so as to selectively destroy one of both nucleotides. This treatment is no longer necessary when using a purified (specific) preparation: either or both nucleotides can then be specifically measured in the presence of a 50-fold or even higher excess of the other.

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