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Stereospecificity of Hydride Transfer and Substrate Specificity for FMN-Containing NAD(P)H-Flavin Oxidoreductase from the Luminescent Bacterium, *Vibrio fischeri* ATCC 7744

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The stereospecificity of the hydride transfer in NAD(P)H-flavin reductase reaction of *V. fischeri* ATCC 7744 was determined by ^{1}H-NMR spectroscopy using stereospecifically labeled reduced β-nicotinamide adenine dinucleotide (β-NADH). The recombinant flavoenzyme, purified from *E. coli* cells, selectively transferred the pro-*R* hydrogen at the C-4 position of the nicotinamide ring to flavin and is therefore classified as an A-side specific enzyme. Lumiflavin was used for the reductase reaction, but lumichrome and α-NADH were not utilized as electron acceptor and donor, respectively. © 1994 Academic Press, Inc.

NAD(P)H-flavin oxidoreductase (flavin reductase or NAD(P)H dehydrogenase/oxidase, EC. 1. 6. 8) of the luminescent bacterium, *Vibrio fischeri* ATCC 7744, is a flavoenzyme [1], which catalyzes the transfer of hydrogen from NAD(P)H to flavin, yielding reduced flavin and $NAD(P)^+$ [1-4], as follows:

$$NAD(P)H + H^+ + \text{oxidized flavin} \longrightarrow NAD(P)^+ + \text{reduced flavin}$$

Under aerobic conditions, reduced flavin is oxidized non-enzymatically by molecular oxygen, resulting in the formation of hydrogen peroxide and oxidized flavin [5]. In luminous bacteria, the flavin reductase plays an important role in supplying free, reduced FMN ($FMNH_2$) to the luminescence reaction [3, 4].

Recently, the flavin reductase gene of *V. fischeri* ATCC 7744 has been cloned and the primary structure of enzyme deduced from the nucleotide sequence. The enzyme was found to have a amino acid sequence homology with the oxygen-insensitive nitroreductases of various bacteria [2, 6-8] and H_2O_2-forming NADH oxidase of *Thermus thermophilus* HB8 [9], but not with NAD(P)H-flavin oxidoreductase (Fre) of *E. coli* [10]. The catalytic properties of the three related enzymes were identical and this was explained being due to a free, reduced flavin mediated-

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chemical reaction [1]. Thus, the similarities of the primary structure and catalytic property reflect the nature of the catalytic domain and/or the conformation of the protein.

Recombinant flavin reductase of *V. fischeri* ATCC 7744 expressed in *E. coli* cells was previously purified chromatographically in a single step using an affinity column. The purified enzyme bound FMN non-covalently as a prosthetic group in a 1:1 stochiometric ratio and the flavin reductase activity was stimulated over 50 fold by addition of exogenous flavins (FMN, FAD and riboflavin) [1]. The enzyme catalyzed the reduction of flavins using NAD(P)H as electron donor to yield hydrogen peroxide and was also able to transfer the electron from NADH through FMN to various other electron acceptors (viz. ferricyanide, cytochrome c, methylene blue, quinones and nitroaromatic compounds) [1].

The stereospecificity of the hydride transfer in NAD(P)H-dependent oxidoreductase reaction has previously been well investigated [11] and has been divided into the two classes, namely A and B, depending on the specificity of the hydrogen transfer at the C-4 position of the nicotinamide ring. A highly conserved structure of the β1-αA-β2 motif for the NAD-binding domain, which contains three conserved glycine residues with the sequence Gly-Xaa-Gly-Xaa-Xaa-Gly (Xaa: any amino acid residue), has also been reported [12]. However, in the case of the flavin reductase in *V. fischeri*, the consensus sequence did not exist in the primary structure. In order to elucidate the reaction mechanism of the FMN-containing flavin reductase, the stereospecificity of the hydride transfer was determined by ^{1}H-NMR spectroscopy using stereospecifically labeled β-NADH and the substrate specificity of the enzyme was also characterized.

Materials and Methods

Materials

The following materials were obtained from commercial sources: lyophilized yeast alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC. 1. 1. 1. 1, Cat. No. 102717), β-NAD$^+$ free acid, β-NADH disodium salt, β-NADPH tetrasodium salt and FMN monosodium salt, from Boehringer Mannheim, Indianapolis, IN; FAD, Calbiochem. San Diego, CA; 2-propanol-d_8 (99+ atom %D), Aldrich, Milwaukee, WI; D_2O (99.9+ atom %D), Isotec. Inc., Miamisburg, OH; lyophilized *Thermoanaerobium brockii* alcohol dehydrogenase (alcohol:NADP$^+$ oxidoreductase, EC. 1. 1. 1. 2, Cat. No. A8435), α-NADH, riboflavin, lumiflavin, lumichrome, lumazine, luminol, Sigma Co., St. Louis, MO. All other chemicals were of the highest grade commercially available.

Enzyme assay for NAD(P)H-flavin reductase

NAD(P)H-flavin reductase activity was determined by measuring the initial rate of NAD(P)H oxidation at 340 nm (ε = 6.22 x10 $^{-3}$ M^{-1} cm^{-1}) [13] at 22 to 24 ^{o}C using a Hitachi (Tokyo, Japan) Model U1100 spectrophotometer [1]. The standard reaction mixture contained 0.1 mM flavin, 0.1 mM NAD(P)H and the purified enzyme (0.4 μg protein), in 0.5 ml of 50 mM sodium phosphate buffer, pH 7.0, and the mixture was preincubated for 1 min at room temperature. The reaction was initiated by adding 5 μl of 10 mM NAD(P)H or flavin. The concentrations of FMN, FAD and riboflavin were determined spectrophotometrically using the absorption coefficients of 12.6 x 10^3 M^{-1} cm^{-1} (445 nm), 11.3 x 10^3 M^{-1} cm^{-1} (450 nm) and 12.5 x 10^3 M^{-1} cm^{-1} (445 nm), respectively [14]. The stock solution (10 mM: riboflavin, lumiflavin, lumichrome) was freshly prepared in 0.05 M NaOH.

Preparation of recombinant flavin reductase containing FMN

Recombinant flavin reductase containing FMN was purified using Cibacron Blue 3G-A affinity column chromatography from *E. coli* cell extracts, as described previously [1]. The

purified enzyme gave a single band by SDS-PAGE (12 %) and was estimated to be >95 % pure and the measured activity was 70 µmol NADH oxidation/min/mg protein in the presence of 0.1 mM FMN.

^{1}H-NMR analysis

^{1}H-NMR spectra were recorded on a JEOL EX400 (400 MHz) instrument in D_2O at 23 ^oC, using t-BuOH (δ 1.23 ppm) in D_2O as a standard.

Preparation of [4R-^{2}H]NADH

[4R-^{2}H]NADH was prepared as previously described [15]. The reaction mixture contained 7 mM β-NAD$^+$ and 0.25 ml 2-propanol-d_8 in 50 mM sodium phosphate buffer, pD 8. The reaction was initiated by adding 240 units yeast alcohol dehydrogenase (400 units/ mg protein) and kept at 25 ^oC for 4 hr. The conversion to β-NADH was monitored at 340 nm. After over 80 % conversion had taken place, the reaction mixture was evaporated *in vacuo*, and after exchanging twice more with D_2O, the residue was dissolved in D_2O. The stereospecificity and the deuterium content of [4R-^{2}H]NADH was determined by the ^{1}H-NMR spectrum at the signal peaks of δ 2.65, 5.96 and 6.93 ppm (C4-H_S, C6-H and C2-H of the dihydronicotinamide ring of NADH) and 8.18, 8.81, 9.13 and 9.32 ppm (C5-H, C4-H, C6-H, C2-H of the nicotinamide ring of NAD$^+$), respectively. [11, 15]. The deuterium content of [4R-^{2}H]NADH was more than 95 %. Without further purification, [4R-^{2}H]NADH was used for the stereospecificity determination of the flavin reductase.

Determination of the stereospecificity of flavin reductase

The reaction mixture contained 3 mM [4R-^{2}H]NADH in 0.6 ml of 25 mM sodium phosphate-D_2O buffer (pD 8), flavin reductase (4 µg protein) and the respective electron acceptor (10 mM stock solution; 60 µl of FMN, FAD or riboflavin in D_2O, 60 µl of lumiflavin in 0.14 M NaOH-D_2O, 120 µl of potassium ferricyanide in D_2O). After incubation for 40 min at 23 ^oC, ^{1}H-NMR spectra of sample were recorded. The stereospecificity of β-NADH was calculated from the ratio [integrated peak area of C4-H (δ 8.81 ppm)] / [average integrated peak area of C2-H (δ 9.32 ppm) and C6-H (δ 9.13 ppm)].

Results and Discussions

The stereospecifically labeled [4R-^{2}H]NADH was prepared by transferring deuterium from 2-propanol-d_8 to β-NAD$^+$ with yeast NAD$^+$ dependent alcohol dehydrogenase, which is specific for the pro-*R* hydrogen transfer of β-NADH (an A-side specific enzyme) [11, 15]. The hydride transfer specificity of the FMN-containing flavin reductase was determined by ^{1}H-NMR analysis, based on the signal change derived from C4-H of the nicotinamide ring of NAD$^+$. The stereochemical course of the hydride transfer by the enzymes is shown schematically in Fig. 1, and Table I summarizes the results of the hydride specificity of the flavin reductase in the presence of various electron acceptors. The recombinant FMN-containing flavin reductase transferred the pro-*R* hydrogen of [4R-^{2}H]NADH preferably to flavin, resulting the formation of deuterium free β-NAD$^+$. When potassium ferricyanide was used as a final electron acceptor without flavin [1], the pro-*R* hydrogen of β-NADH was also transferred to non-covalently bound-FMN in the enzyme. Under auto-oxidative conditions (heat treatment) with potassium ferricyanide and [4R-^{2}H]NADH in a control experiment [11], deuterium ion exchange between D_2O and β-NAD$^+$ occurred non-enzymatically. Therefore, the FMN-containing flavin reductase of *V. fischeri* was classified as an A-side specific enzyme. Further, [4R-^{2}H]NADPH was prepared by using NADP$^+$ dependent alcohol dehydrogenase from *T. brockii*, which is classified as an A-side specific enzyme [16,17]. Using FMN, FAD and riboflavin (1 mM), the flavin reductase (12 µg/ml) was incubated with 3

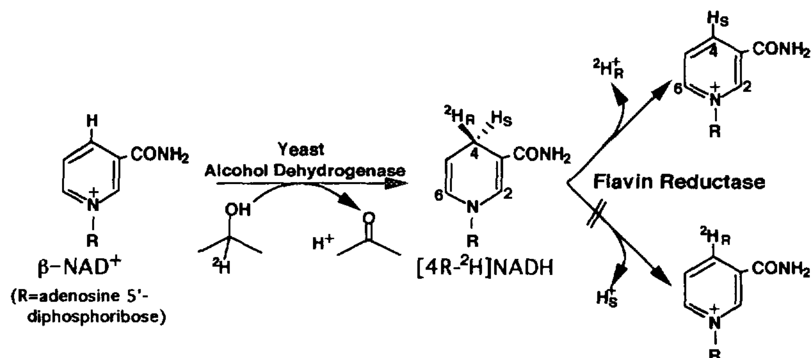

Figure 1. Schematic representation of the stereochemical course catalyzed by *V. fischeri* FMN-containing flavin reductase.

mM [4R-^{2}H]NADPH for 1.5 hr and the stereospecificity of the flavin reductase was determined by ^{1}H-NMR analysis using the same procedures. The specificity of NADPH toward FMN, FAD and riboflavin was found to be 80 %, 76 % and 74 %, respectively, where the conversion ratio of NADPH to NADP^{+} was 83 %, 60 % and 70 %, respectively. Though the reason for the lower specificity of NADPH than NADH is not clear, the flavin reductase favorably transferred the pro-*R* hydrogen of NADPH to flavin, the same as in the case of NADH.

The stereospecificity of another flavin reductase from the luminescent bacterium, *Vibrio harveyi*, has been determined using crude extracts and the enzyme was classified as an A-side specific enzyme [18]. Later, in *V. harveyi*, it was reported that NADH-, NADPH- and NAD(P)H-dependent flavin reductase are present [19-23] and two different flavin reductase genes have been recently cloned [24-26]. The primary structure and catalytic properties of NAD(P)H-flavin reductase from *V. fischeri* [1, 2] was found to be similar to that of NADPH-flavin reductase from *V. harveyi* [26] (data not shown).

As shown in Fig. 2, various flavins (1-3) and related compounds (4, 5) were examined as electron acceptor for the flavin reductase reaction. Table II summarizes the substrate specificity of

Table I. Stereospecificity of flavin reductase

Substrate	Conc.	% H at C4-H of NAD^{+}	Stereo-Specificity
FMN	1 mM	> 82 (100)$^{a)}$	H_R
FAD	1 mM	> 90 (100)	H_R
Riboflavin	1 mM	> 88 (100)	H_R
Lumiflavin	1 mM	> 88 (60)	H_R
Ferricyanide	2 mM	> 86 (56)	H_R
Ferricyanide$^{b)}$	6 mM	35 (50)	-

The reaction conditions are described in Materials and Methods. The calculation of % H at C4-H of the nicotinamide ring of NAD^{+} was carried out using the ratio: [integrated peak area of C4-H] / [average integrated peak area of C2-H and C6-H]. a) Parentheses indicate the percentage of conversion from NADH to NAD^{+}. b) Heat treatment at 80 ^{o}C for 1 min without enzyme.

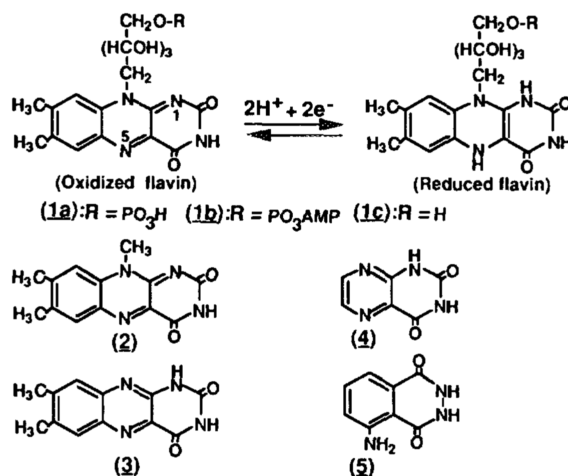

Figure 2. Chemical structure of flavins and related compounds: (1a) FMN, (1b) FAD, (1c) riboflavin, (2) lumiflavin, (3) lumichrome, (4) lumazine, (5) luminol.

the FMN-containing flavin reductase. FMN (1a), FAD (1b) and riboflavin (1c) were used as a suitable electron acceptor for the flavin reductase reaction [1] and β-NADH was a preferable electron donor than β-NADPH. The flavin reductase did not utilize α-NADH in stead of β-NAD(P)H as an electron donor for the flavin reduction. It was noteworthy that lumiflavin (2) was used as electron acceptor for the β-NAD(P)H reduction reaction (Table I and II), but lumichrome (3) was not used for the reaction. On the other hand, the flavin reductase activity was strongly inhibited by lumazine (4) and luminol (5), which have a structural similarity to isoalloxazine moiety of flavin. The inhibition of lumazine and luminol seems to be competition with flavin (isoalloxazine ring) binding to the enzyme. Thus, the binding of the isoalloxazine ring apparently causes the protein to be activated, allowing for a reduced molecule of β-NAD(P)H to be bound and pro-*R* hydrogen to be transferred to flavin.

The K_m, V_{max} and K_{cat} values for riboflavin, FMN and FAD, in the presence of either β-NADH or β-NADPH, were determined by the method of Lineweaver and Burk [26] (Table III).

Table II. Substrate specificity of flavin reductase

Substrates	Relative Initial Activity (%)			
	β-NADH		β-NADPH	α-NADH
	I a)	II b)		
FMN (1a)	100	-	30	0
FAD (1b)	76	-	31	0
Riboflavin (1c)	84	-	21	0
Lumiflavin (2)	20	88	5	0
Lumichrome (3)	0	88	0	-
Lumazine (4)	0	14	0	-
Luminol (5)	0	0	0	-

a) NAD(P)H-flavin reductase assay was performed as described in Materials and Methods. b) NADH-FMN reductase activity after incubation for 1 min with various compounds (0.1 mM).

Table III. Kinetic analyses of flavin reductase

Electron Donors/Acceptors	Substrates	Km (μM)	Vmax (μM/min)	kcat (sec^{-1})
a) NADH	FMN	19.3	88.5	46.1
	FAD	32.3	68.2	35.2
	Riboflavin	8.6	91.9	47.9
b) NADPH	FMN	9.2	29.9	15.6
	FAD	10.1	21.5	11.2
	Riboflavin	1.9	30.5	15.9
c) FMN	NADH	32.7	89.0	45.8
	NADPH	24.2	40.5	21.2
d) FAD	NADH	23.5	66.3	34.5
	NADPH	34.9	22.4	11.7
e) Riboflavin	NADH	40.5	61.4	32.0
	NADPH	26.1	13.5	7.0

The concentration of flavin reductase was 32 nM (0.4 μg) and the donor/acceptor concentrations for NAD(P)H and flavins were 100 μM and 60 μM, respectively. The substrate concentration varied from 2.5 μM to 100 μM.

At the same time, K_m, V_{max} and K_{cat} values for β-NADH and β-NADPH toward riboflavin, FMN and FAD were also determined. Since a strong inhibition of reductase activity was observed at high concentrations (over 200 μM) of either flavin or β-NAD(P)H under the standard reaction condition, the determination were carried out at substrate concentrations of 2.5 μM to 100 μM. Using the FMN-containing flavin reductase in combination with either β-NADH or β-NADPH as electron donor, the K_{cat} values for flavins in the presence of β-NADH were found to be three times higher than in the presence of β-NADPH, indicating a preference for β-NADH in the reaction. Further, FMN also serves as a suitable substrate for the reductase reaction. No significant difference in K_{cat} values between β-NADH for FMN, and FMN for β-NADH, was observed, suggesting that the turnover numbers for reduction of FMN and oxidation of β-NADH are almost equal in the NADH-FMN reductase reaction. These results, taken together with the NAD(P)H-stereospecificity and the substrate specificity of the enzyme, suggested that the stereochemical course of the reaction was exactly controlled by the flavin reductase.

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References

1. Inouye, S. (1994) FEBS Lett. 347, 163-168.
2. Zenno. S, Saigo, K., Kanoh, H. and Inouye, S. (1994) J. Bacteriol. 176, 3536-3543.
3. Baldwin, T. O. and Ziegler, M. (1992) In Chemistry and Biochemistry of Flavoenzymes (Müller, F. ed.) vol. III, pp467-530. CRC Press, Inc. Florida.
4. Meighen, E. A. and Dunlap, P. V.(1993) Adv. Microbiol. Physiol. 34, 1-67.
5. Gibson, Q.H. and Hastings, J.W. (1962) Biochem. J. 83, 368-377.
6. Watanabe, M., Ishidate, M, and Nohmi, T. (1990) Nucl. Acids Res. 18, 1059.
7. Bryant, C., Hubbard, L. and McElroy, W. D. (1991) J. Biol. Chem. 266, 4126-4130.
8. Anlezark, G.M., Melton, R.G., Sherwood, R.F., Coles, B., Friedlos, F., and Knox, R.J. (1992) Biochem. Pharmacol. 44, 2289-95.
9. Park, H. -J., Kreutzer, R., Reiser, C. O. A. and Sprinzl, M. (1992) Eur. J. Biochem. 205, 875-879. Corrections 211, 909.

10. Spyrou, G., Haggard-Ljungquist, E., Krook, M., Jornvall, H., Nilsson, E. and Reichard, P. (1991) J. Bacteriol. 173, 3673-3679.
11. You, K. (1984) CRC Crit. Rev. Biochem. 17, 313-451.
12. Branden, C. and Tooze, J. (1991) In Introduction to Protein Structure, p141-159. Garland Publishing, Inc. New York.
13. Horecker, B. L. and Kornberg, A. (1948) J. Biol. Chem. 175, 385-390.
14. Whitby, L. G. (1953) Biochem. J. 54, 437-442.
15. Mostad, S. B. and Glasfeld, A. (1993) J. Chem. Educ. 70, 504-506.
16. Ottolina, G., Riva, S., Carrea, G., Danieli, B. and Buckmann, A.F. (1989) Biochim. Biophys. Acta 998, 173-178.
17. Wong, S. S. and Wong, L-J. C. (1983) Int. J. Biochem. 15, 147-150.
18. Fisher, J. and Walsh, C. (1974) J. Amer. Chem. Soc. 91, 4345-4346.
19. Gerlo, E. and Charlier, J. (1975) Eur. J. Biochem. 57, 461-467.
20. Michaliszyn, G. A., Wing, S. S. and Meighen, E. A. (1977) J. Biol. Chem. 252, 7495-7499.
21. Jablonski, E. and DeLuca, M. (1977) Biochemistry 16, 2932-2936.
22. Jablonski, E. and DeLuca, M. (1978) Biochemistry 17, 672-678.
23. Watanabe, H. and Hastings, J. W. (1982) Mol. and Cell. Biochem. 44, 181-187.
24. Izumoto, Y., Mori, T. and Yamamoto, K. (1994) Biochim. Biophys. Acta. 1185, 243-246.
25. Zenno, S. and Saigo, K. (1994) J. Bacteriol. 176, 3544-3551.
26. Lei, B., Liu, M., Huang, S. and Tu, S.-C. (1994) J. Bacteriol. 176, 3552-3558.
27. Segel, I. H. (1975) In Enzyme kinetics, p46-49. John Wiley and Son Inc., New York.