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COMPARISON OF COENZYME F_{420} FROM METHANOBACTERIUM BRYANTII WITH 7- AND 8-HYDROXY-10-METHYL-5-DEAZAISOALLOXAZINE

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SUMMARY

Syntheses of 7-hydroxy-10-methyl-5-deazaisoalloxazine (7-HMDI) and 8-hydroxy-10-methyl-5-deazaisoalloxazine (8-HMDI) are described. The physicochemical and biological properties of 8-HMDI, in contrast to those of 7-HMDI, are very analogous to those of F_{420}, a coenzyme found in methanogenic bacteria.

INTRODUCTION

In the proposed structure of F_{420} (the N-(N-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavine-5-phosphate) from Methanobacterium bryantii the structure of the heterocyclic ring (8-hydroxy-5-deazaisoalloxazine) was assigned on the basis of NMR spectra and comparison of its UV spectrum with that of 8-hydroxy-FMN (1). Some uncertainty remained about the position of the hydroxy group, which was most probably located at carbon-8, but a carbon-7 position could not be excluded. To obtain unequivocal proof both 7- and 8-hydroxy-10-methyl-5-deaza-isoalloxazine (7-HMDI and 8-HMDI) were synthesized and their physicochemical and biological properties compared with those of F_{420}. While this study was in progress Ashton et al. (2) reported on the synthesis and biochemical properties of 7,8-didemethyl-8-hydroxy-5-deazariboflavine and 8-demethyl-8-

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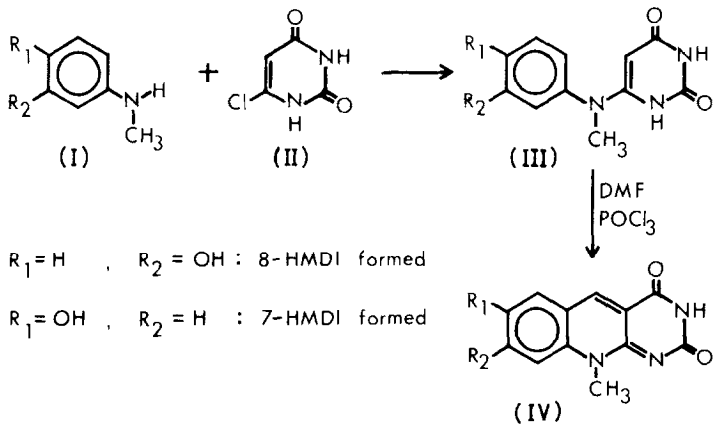

hydroxy-5-deazariboflavin·, but they did not include a 7-hydroxy derivative in the comparative study of these compounds with FO, a hydrolytic derivative of F_{420}.

MATERIALS AND METHODS

Synthesis of 7- or 8-HMDI was performed in analogy to the method of Yoneda et al. (3) by mixing para- or meta-hydroxy-N-methylaniline (I) with 6-chlorouracil (II) and heating for half an hour at 120°C. Crystallization of the products leaves colourless crystals of (III) with melting points at 335°C and 312°C (decomp.), respectively. The obtained products were dissolved in dimethylformamide (DMF) and treated with $POCl_3$ at 0°C. Stirring for one hour and heating then at 100°C for half an hour gave 7-HMDI as orange and 8-HMDI as yellow crystals (IV) with melting points above 360°C.

Proton NMR spectra were obtained on a Bruker, model WH-90 spectrometer, UV-visible spectra on a Cary model 118 spectrophotometer and fluorescence data on an Aminco Bowman spectrofluorimeter. pK_a values were estimated spectrophotometrically as described previously (1). Redox measurements were performed by potentiometric titration in a three-armed anaerobic cuvet equipped for anaerobic addition of gases and liquids, and for simultaneous measurement of the absorbancy at the appropriate wavelength and redox potential with a calomel and platinum electrode. Janus green (-258 mV), neutral violet (-340 mV), riboflavin (-208 mV) and H_2 (-421 mV) were used as reference. 7-HMDI and 8-HMDI were reduced by H_2 and cell-free extract of M. bryantii and after replacement of H_2 with oxygen-free N_2, oxidation was performed by anaerobic titration with ferricyanide or oxygen. The pH was kept constant with 50 mM phosphate buffer.

M. bryantii was grown as described previously (4). Preparation of cell-free extract and the assays of hydrogenase and NADP-linked hydrogenase were performed as reported earlier (5).

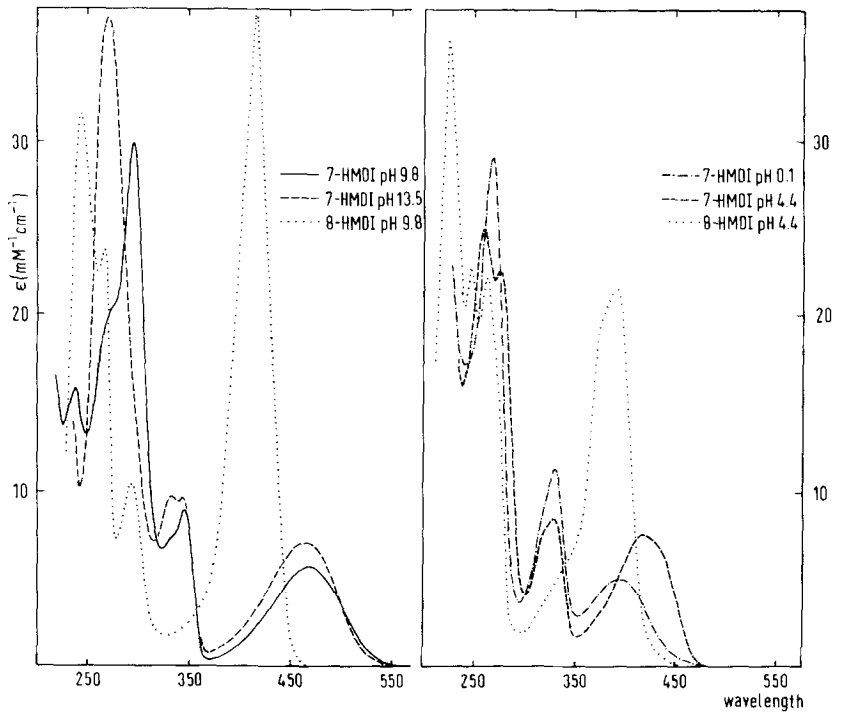

Fig. 1. UV-visible spectra of 7- and 8-HMDI

RESULTS AND DISCUSSION

Fig. 1 presents the UV-visible spectra of 7-HMDI in its four ionic forms. These spectra differ strongly from those of 8-HMDI, which in turn appeared to be identical to those of F_{420}, apart from blue shifts up to 3 nm and molar extinction coefficients 0.6-0.7 times those of F_{420} (Table I). Whereas reduced 8-HMDI and F_{420} have an identical absorption band at 320 nm, reduced 7-HMDI absorbed maximally at 313 nm; both values were measured at pH 7.0. Striking differences were observed between 7-HMDI on one hand and 8-HMDI and F_{420} on the other hand as to the fluorescent properties, the pK_a values of the hydroxy group (8.2 and near 6.0), proton NMR spectra and the redox potentials. Therefore, the physicochemical properties of 8-HMDI, in contrast to those of 7-HMDI, are very consistent with those of F_{420}.

Table I. Physicochemical properties of 7-HMDI, 8-HMDI and F_{420}.

		7-HMDI		8-HMDI		F_{420}[f]	
Proton NMR[a]		in DMSO	in D_2O[b,c]	in DMSO	in D_2O[b,d]	in D_2O[b,e]	
		8.92(s)	8.13(s) H_5	9.13(s)	7.71(s) H_5	7.92(s)	H_5
		7.51(m)	6.73(d) H_6	8.20(d)	7.22(d) H_6	7.29(d)	H_6
		7.51(m)	7.29(s) H_8	7.32(d)	6.68(d) H_7	6.61(d)	H_7
		7.83(d)	7.29(s) H_9	7.43(d)	6.22(d) H_9	6.45(s)	H_9
		4.02(s)	3.72(s) CH_3	4.02(s)	3.49(s) CH_3		
UV-visible properties λ_{max} (ϵ) nm ($mM^{-1}cm^{-1}$)	pH 0.1	225(22.2)	270(28.5)	228(26.7)	248(15.8)	230(39.7)	250(22.6)
		330(11.0)	394(4.8)	264(12.9)	374(22.8)	267(21.2)	375(32.8)
	pH 4.4	227(22.9)	261(24.6)	232(33.8)	249(21.5)	235(41.5)	250(24.0)
		276(22.2)	329(8.3)	264(20.9)	393(20.6)	267(25.4)	395(27.4)
		418(7.4)					
	pH 9.8)	236(15.7)	292(29.3)	245(32.0)	265(24.0)	247(37.0)	267(25.8)
		345(8.8)	468(5.5)	294(10.9)	417(37.1)	295(12.6)	420(45.5)
	pH13.5	272(36.9)	332(9.7)	244(43.3)	287(9.2)	245(54.6)	290(11.4)
		344(9.6)	464(7.1)	418(41.5)		420(54.3)	
pK_a values chromophore		2.1 8.2 11.6		1.9 6.0 12.1		1.7 6.3 12.2	
Redox potential (mV)		-275 (pH 8.0)		-350 (pH 7.0)		-340 (pH 7.0)	
Fluorescence[g]	pH 4.4	480 (430), 0.15%		440 (390), 36%		460 (390), 46%	
	pH 9.8	N.D.		467 (417), 76%		470 (420),100%	

a, Values are given as shifts from TMS in ppm. b, D_2O containing 5% Na_2CO_3, 7-HMDI is slightly soluble in this solvent, therefore KOD was added (0.1 N). c, $J_{6,8}$ = 2 Hz. d, $J_{6,7}$ = 9.0 Hz, $J_{7,9}$ = 1.5 Hz. e, Aromatic part of F_{420} only. f, Except for the redox potential and fluorescence, data are taken from reference 1. g, Fluorescence data are given as maximal emission wavelength (nm) and within parentheses the activation wavelength (nm). The intensities, measured at equimolar concentrations, are compared with that of F_{420} at pH 9.8 (100%). The wavelengths may deviate 5 nm, the intensities 10% of the actual values. N.D. = fluorescence not detectable.

Hydrogenase present in cell-free extract of M. bryantii reduced 7-HMDI and 8-HMDI with apparent K_m-values of 65 and 100 μM and V_{max} 0.7 and 0.3 times that of F_{420}, respectively. These values compare very well with the values (within parentheses) found for F_{420} (25 μM) and its hydrolytic fragments FO (100 μM) and F^+ (100 μM). In spite of the fact that hydrogenase appears to react in a rather aspecific way as to the electron acceptor used, in accordance with the results reported previously (5), the rather low K_m-values for deazaisoalloxazines may indicate that they are preferred substrates. Chemically reduced 7-HMDI, 8-HMDI and F_{420} were only slowly oxidized by oxygen in accordance to observations with 5-deazaflavins (6). However, when reduced by cell-free extract of M. bryantii, reduced 8-HMDI and F_{420} can be partly oxidized by the addition of oxygen, but the reoxidation rate became very slow when redox potentials higher than about -340 mV were reached. These results suggest that hydrogenase can use reduced 8-HMDI and F_{420} as electron donors and oxygen as electron acceptor. This may reflect a way in which methanogens can remove traces of oxygen, which is toxic for these strictly anaerobic organisms. Possibly due to oxidation of the enzyme the enzymatic function of hydrogenase is inhibited at redox potentials higher than about -340 mV. Moreover this inhibiting effect could explain why 7-HMDI, with a redox potential of -275 mV, cannot be reoxidized in this way. Reoxidation of 7-HMDI could be performed with ferricyanide, which is rather surprising because this oxidant is known to react very slowly with deazaflavins (6).

8-HMDI could replace F_{420} in the NADP-linked hydrogenase assay which was found to be specific for F_{420} and some

closely related derivatives (5,7). Probably due to the high redox potential as compared to NADP (-320 mV) 7-HMDI could not replace F_{420} in this assay. 7-HMDI inhibited the 8-HMDI mediated NADP reduction slightly: a 10% inhibition was observed when a 4-fold molar excess of 7-HMDI, as compared to 8-HMDI, was used. So the NADP-linked hydrogenase assay differentiates between 7-HMDI on one hand and 8-HMDI and F_{420} on the other hand.

From the physicochemical and biological properties of 7- and 8-HMDI it may be concluded that the structure assignment of F_{420} as a 8-hydroxy derivative of 5-deazaisoalloxazine was correct.

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