Effect of pH on Oxidation–Reduction Potentials of 8α-*N*-Imidazole-Substituted Flavins†

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ABSTRACT: The pK_a values for the various ionic forms of 8α-*N*-imidazolylriboflavin were determined in its oxidized and hydroquinone forms and estimated for its semiquinone form. The pH dependence of the absorption and fluorescence spectral properties and potentiometric titration data show the pK_a values for the oxidized form to be 6.02 ± 0.03 for the 8α-imidazole nitrogen and 9.67 ± 0.05 for the N(3) position of the flavin ring. The pH dependence of the oxidation–reduction potential was determined by spectrocoulometric titrations, and the data points were compared with computer-simulated plots. Two pK_a values for the hydroquinone form of the flavin were determined and assigned. The pK_a for the imidazole ring is found to be 6.9 ± 0.1 and for the N(1) position of the flavin hydroquinone is found to be 5.5 ± 0.1. Analysis of the pH dependence of the one-electron couples E_2 (flavoquinone/flavin semiquinone, $Fl_{ox}/Fl\cdot$) and E_1 (flavin semiquinone/flavin hydroquinone, $Fl\cdot/Fl_{red}$) resulted in an estimated pK_a of 6.5 for the 8α-imidazole ring in the flavin semiquinone form. These data show the possible involvement of the ionization of the 8α-imidazole substituent in the redox chemistry of flavoenzymes containing either an 8α-N^1- or an 8α-N^3-histidyl-linked covalent flavin coenzyme. Future work on oxidation–reduction potentials of this class of enzymes must take into consideration the influence of the 8α-histidyl substituent.

Since the original elucidation of the structure of the covalently bound flavin of mitochondrial succinate dehydrogenase as 8α-N^3-histidyl-FAD (Walker et al., 1972; Salach et al., 1972), approximately 25 different enzymes from a variety of sources have been shown to contain covalent flavin residues (Singer & McIntire, 1984). Of these 25 enzymes, 15 have been shown to contain histidyl substituents linked to the 8α position of the flavin at either the N(3) or the N(1) positions of the imidazole ring (Singer & McIntire, 1984).

There has been little information or insight with regard to how the presence of an 8α substituent such as histidine could influence the oxidation–reduction properties (and thus the catalytic function) of the enzyme-bound flavin. Previous work has shown the 8α-histidyl substituent to have only small effects on the oxidation–reduction potential (E_m = 7) and sulfite affinity of the flavin ring (Edmondson & Singer, 1973; Edmondson et al., 1976). Flash photolysis studies (Edmondson et al., 1977) have shown that the presence of an 8α-histidyl substituent decreases the pK_a of the neutral semiquinone form of the flavin by over 1 unit as compared with riboflavin. Nothing is known to date with regard to the effect of flavin reduction on the ionization behavior of the 8α-imidazole ring. The pK_a is known to be decreased (relative to free histidine) in the oxidized form of 8α-histidylflavins as judged from alterations in absorbance and fluorescence of the flavin moiety on deprotonation of the 8α-imidazole ring.

Recent studies on the pH dependence of the oxidation–reduction potential of the 8α-N^3-histidyl-FAD of succinate dehydrogenase (Ohnishi et al., 1981) have suggested pK_a values for the N(5) position of the flavin semiquinone to be 8.0 and for the N(1) position of the flavin hydroquinone to be 7.7. A pK_a of 8.0 for the flavin semiquinone is difficult to reconcile with electron spin resonance (ESR) data (Edmondson et al., 1981), which shows the flavin semiquinone form of succinate dehydrogenase to exhibit the ESR spectral properties of the anionic form in the pH range of 6.1–9.1. Possible explanations by Ohnishi et al. (1981) to correlate the ESR data with the oxidation–reduction potential data on succinate dehydrogenase include the possibility of an altered distribution of spin density in the flavin as compared with 8α-histidylflavin peptides bound to flavodoxin (Edmondson et al., 1981) or the protonation–deprotonation of a neighboring amino acid residue.

Before any pH-dependent redox potential data on proteins containing 8α-histidylflavins can be interpretable, it is essential to study the pH dependence of the redox potential of a model flavin analogue. This paper reports such a study on synthetic 8α-*N*-imidazolylriboflavin. This particular analogue was chosen rather than 8α-N^3- or 8α-N^1-histidylriboflavin to eliminate any complications arising from the ionization of the α-amino or carboxyl groups of the amino acid substituent and any complications arising from interaction of the side chain of the flavin with the histidyl side chain of the 8α-N^1-histidylriboflavin (Edmondson et al., 1976). The results described in this paper on the use of electrochemical techniques show that the pK_a of the 8α-imidazole substituent increases by 1 pH unit on reduction of the flavin to its hydroquinone level. Thus, any studies on pH-dependent redox potentials of enzymes containing an 8α-histidyl-substituted flavin must take into consideration the ionization of the imidazole ring as well as positions on the flavin ring.

Experimental Procedures

Synthetic Flavins. 8α-*N*-Imidazolylriboflavin was synthesized in a manner analogous to the synthesis of 8α-N^3-histidylriboflavin (Walker et al., 1972). 8α-Bromotetra-*O*-acetylriboflavin was synthsized as described by Walker et al. (1972) and incubated (4 g, 5.8 mmol) with a 5-fold molar excess of imidazole in 15 mL of dimethylformamide at 25 °C for 24 h. The flavin was precipitated by the addition of cold diethyl ether, the residue was dissolved in $CHCl_3$ and extracted into 0.1 M potassium phosphate, pH 3.5. The pH of the aqueous flavin solution was then adjusted to 7.5 by the addition of solid Na_2CO_3, and the flavin was reextracted into $CHCl_3$.

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The $CHCl_3$ solution was dried with Na_2SO_4, evaporated to dryness on a rotary evaporator, and further dried in vacuo. Complete hydrolysis of the acetyl groups from the flavin side chain was achieved by refluxing for 15 min in 0.2 N HCl. The solution was concentrated by rotary evaporation to ~30 mg/mL and the flavin precipitated by adjusting the pH to 7.5 with dilute NaOH. Pure 8α-*N*-imidazolylriboflavin was obtained by the addition of ice-cold ethanol (20 volumes) to an aqueous flavin solution (10–20 mg/mL) at pH 3.0. The precipitated flavin was collected by filtration, dried in vacuo, and stored desiccated at −20 °C in the dark. The product was homogeneous by TLC, R_f (solvent 1) 0.1 and R_f (solvent 2) 0.01, and by HPLC. The absorption spectrum at pH 7.0 showed maximal absorption at 268.5 (ϵ = 34 500 M^{-1} cm^{-1}), 355 (ϵ = 8800 M^{-1} cm^{-1}), and 445 nm (ϵ = 11 600 M^{-1} cm^{-1}) and at pH 3.7 showed maximal absorption at 368 (ϵ = 35 100 M^{-1} cm^{-1}), 346 (ϵ = 8700 M^{-1} cm^{-1}), and 445 nm (ϵ = 11 300 M^{-1} cm^{-1}). ^1H NMR spectral data of the hydrochloride salt (360 MHz, D_2O) showed the following: δ 2.47 (s, 3 H), 3.6–5.0 (m, 7 H), 5.9 (s, 2 H), 7.59 (s, 1 H), 7.98 (s, 1 H), 7.76 (s, 1 H), 7.98 (s, 1 H), and 8.93 (s, 1 H).

8α-(*N*-Methyl-*N*-imidazolium)tetra-*O*-*N*-acetylriboflavin was synthesized by incubation of 8α-*N*-imidazolyltetra-*O*-acetylriboflavin with a 5-fold molar excess of CH_3I in dimethylformamide for 10–12 h. The reaction was followed until the fluorescence at pH 7.5 was equivalent to that at pH 3.5, which showed complete alkylation of the 8α-imidazole substituent. The flavin was precipitated by the addition of cold diethyl ether, the residue dissolved in methanol and reprecipitated by the addition of cold ether, and the product dried in vacuo and stored desiccated in the dark. The product was homogeneous by TLC: R_f (solvent 1) 0.17 and R_f (solvent 2) 0.48. HPLC analysis showed the presence of small amounts of mono-, di-, and trideacylated material. The absorption spectrum at pH 4.1 and at 7.0 was identical: maximal absorption was at 268 (ϵ = 35 900 M^{-1} cm^{-1}), 343 (ϵ = 8970 M^{-1} cm^{-1}), and 445 nm (ϵ = 11 800 M^{-1} cm^{-1}).

The proton NMR analysis of the 8α-*N*-imidazolylriboflavin will be extensively described in a forthcoming paper (Williamson & Edmondson, 1985). The *N*-methyl proton resonance of 8α-(*N*-methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin is observed as a three-proton singlet at 3.98 ppm.

Thin-layer chromatography of the synthetic flavins was performed on Whatman LK6DF 40-Å silica gel plates in 1-butanol–acetic acid–water (2:1:1 v/v/v) (solvent 1) or chloroform–methanol–acetic acid (18:1:1 v/v/v) (solvent 2). Flavin spots were visualized by their fluorescence under an UV light.

Methods. Spectrocoulometric titrations were performed at 25 °C in a cell similar in design to that described by Stankovich (1980). Reducing or oxidizing potential was applied to the stirred flavin solution with an ECO Model 553 potentiostat, and the total number of coulombs added to the system was measured with an ECO Model 731 digital integrator. A coil of gold wire served as the working electrode, and a silver wire in a KCl solution (separated from the flavin solution by a thin layer of "thirsty glass") served as the counter electrode. Solution potentials were measured with a Pt electrode relative to a Ag/AgCl double-junction reference electrode (Tacussel, Model RDJ-10, Astra Scientific Co.) in an Orion Model 801 pH/mV digital meter. Absorption spectra during the redox titrations were measured with a Cary 14R spectrophotometer equipped with a thermostated cell holder and a magnetic stirrer situated beneath the cell holder (Aviv and Associates).

In a typical titration, a flavin solution (protected from light at all times) in 0.1 M buffer containing 0.1 mM methyl viologen and 0.1 mM potassium ferrocyanide was made anaerobic by at least eight cycles of alternate degassing under vacuum and flushing with argon that had been purified from any residual O_2 by passage through a BASF catalyst at 140 °C (Williams et al., 1979). Measured current efficiencies of ~95% were found, and potentials determined on the reoxidative phase of the titration were always in excellent agreement with those recorded during the reductive phase. The reference electrode was standardized before each titration with a deoxygenated, saturated quinhydrone solution in 0.09 M KCl/0.01 M HCl at 25 °C (O'Reilly, 1973; Bates, 1973). The potential of this system is 575.5 mV vs. the standard hydrogen electrode. Typically, the Ag/AgCl reference electrode gave a measured potential of 201.4 ± 0.7 mV (seven determinations) when standardized daily. Fresh KCl solutions were used when the reference potential deviated from this value by more than 10 mV.

Data Analysis. Oxidation–reduction potential data were fitted to the Nernst equation with a linear least-squares regression program in which all data points are given equal weight. All data computations were performed with BASIC programs written in this laboratory and run on a Northstar Horizon microcomputer. The potentials for the flavosemiquinone/flavohydroquinone couple (E_1) and of the flavoquinone/flavosemiquinone couple (E_2) were calculated from an iterative best fit of the data to the Michaelis equation as described previously (Draper & Ingraham, 1968):

$$E_h = E_m + \frac{RT}{2F} \ln \frac{(1+\mu)}{(1-\mu)} + \frac{RT}{2F} \ln \frac{[1+\gamma(1-\mu^2)]^{1/2}+\mu}{[1+\gamma(1-\mu^2)]^{1/2}-\mu}$$

This equation relates the flavin redox potential (E_h) to the semiquinone formation constant (K) where $\gamma = 40K - 1$ and the degree of oxidation (μ) is scaled from −1 (100% reduced) to 1 (100% oxidized) (Clark, 1960; Draper & Ingraham, 1968). E_1 and E_2 are calculated from $E_2 - E_1 = 0.05916 \log K$. An estimation of the error in K is obtained from the standard deviation of the Nernst plot by measurement of the "index potential" (E_i) (Michaelis, 1932; Michaelis et al., 1937).

pK_a values from the pH dependence of E_m were estimated by the agreement of experimental data points with a computer-generated theoretical plot of the equation

$$E_m = E_0 - 0.05916\text{pH} + \frac{RT}{NF} \ln \frac{[H^+]^2 + K_r[H^+] + K_{r1}K_{r2}}{[H^+]^2 + K_{o1}[H^+] + K_{r1}K_{o1}}$$

which describes the relationship between E_m, pH, and two pK_a values for the oxidized form and two pK_a values for the reduced form of the flavin (Clark, 1960). In systems such as riboflavin in which the oxidized form has one pK_a value [the N(3) position] and the reduced form only one pK_a [the N(1) position], the pK_a values can be satisfactorily obtained by graphical procedures (Draper & Ingraham, 1968). Where more complex ionizations are involved such as with 8α-*N*-imidazolylflavins, a fit to the computer-generated theoretial curves of the above equation is essential for proper analysis.

Results

The structures of the various ionic forms of 8α-*N*-imidazolylriboflavin that could exist in the oxidized, semiquinone, and hydroquinone forms are shown in Scheme I. The structures indicated are those expected in the pH range be-

Scheme I

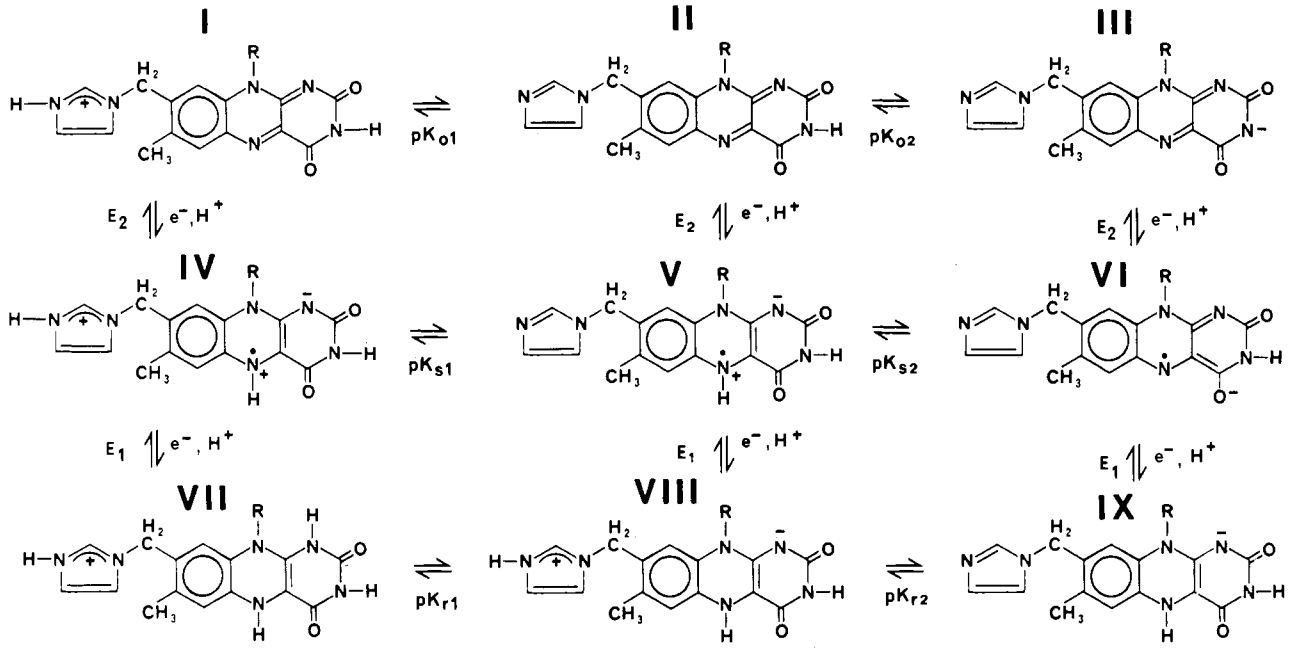

Table I: Determination of pK_a Values for Oxidized 8α-N-Imidazolylriboflavin

method of determination	pK_{o1} (imidazole)	pK_{o2} [flavin N(3)]
potentiometric titration[a]	6.04 ± 0.04	9.67 ± 0.03
pH dependence of fluorescence quenching	5.99 ± 0.03	9.72 ± 0.03
pH dependence of λ_{max}	6.02 ± 0.02	9.62 ± 0.03
mean values	6.02 ± 0.03	9.67 ± 0.05

[a] Potentiometric titrations were performed as described by Albert & Serjeant (1984).

tween 2.5 and 11.5, the pH range investigated in this study. Only one tautomeric form is presented for each ionic species although it is recognized that other tautomeric species could be present.

Determination of pK_{o1} and pK_{o2} of 8α-N-Imidazolylriboflavin. To assist in the interpretation of the pH dependence of the oxidation–reduction potential data, it was first necessary to determine the pK_a values of the imidazole flavin in its oxidized form. The two pK_a values in question would be that of the unsubstituted nitrogen in the imidazole ring (pK_{o1}) and the N(3) position of the flavin ring (pK_{o2}). Unlike riboflavin, both the position of the near-ultraviolet absorption band and the fluorescence yield of 8α-N-histidylflavins are altered in the pH range between 3 and 8 (Walker et al., 1972) due to ionization of the imidazole ring. 8α-N^1-Histidylriboflavin has a pK_a of 5.2 (Edmondson et al., 1976) while 8α-N^3-histidylriboflavin exhibits a pK_a of 4.7 (Walker et al., 1972). The pK_a for imidazole ionization of 8α-N-imidazolylriboflavin (pK_{o1}) is found to be 6.02 ± 0.03 as judged from the pH dependence of the visible absorption spectrum, from fluorescence yield, and by potentiometric titration (Table I). In a manner similar to 8α-N-histidylflavins, the absorption maximum shifted from 346 nm at pH 3 to 355 nm at pH 8. The flavin fluorescence yield decreased by 85% on imidazole deprotonation. Linear Henderson–Hasselbalch plots were obtained from both absorption and fluorescence data, and the pK_a values determined by these spectral techniques are in excellent agreement with the potentiometric titration data (Table I).

The N(3) position of the isoalloxazine ring of riboflavin has been determined to have a pK_a of 10.0 (Draper & Ingraham, 1968). The corresponding pK_{o2} for 8α-N-imidazolylriboflavin is determined to be 9.67 ± 0.05 by absorption, fluorescence, and potentiometric titrations (Table I). Thus, the presence of an 8α-imidazole substituent decreases the pK_a of the N(3) position of the isoalloxazine ring by ~0.3 pH unit.

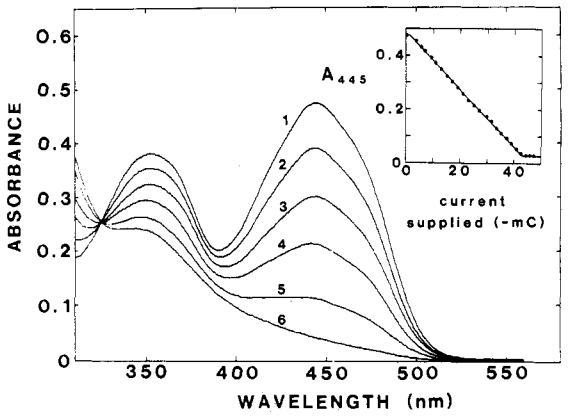

FIGURE 1: Spectrocoulometric reduction of 8α-N-imidazolylriboflavin. Flavin (41 μM) in 5.3 mL of 0.1 M Tris-HCl buffer, pH 8.39 (curve 1), was reduced by an applied potential of −460 mV. Curves 2–5 are intermediates in the reduction, and curve 6 is fully reduced flavin after the addition of 44 mC (inset). A further 10 mC partially reduced the methyl viologen, with no apparent further change in the flavin spectrum.

Assessment of the Spectrocoulometric Technique in the Determination of Flavin Redox Potential. To achieve the maximal accuracy possible, it is important to check independently the method to eliminate any artifacts that may arise due to flavin aggregation, flavin–methyl viologen interactions, etc. The current efficiency of the apparatus was determined to be 95% as judged from the number of millicoulombs required for reduction of a known quantity of flavin (Figure 1) on the basis of the extinction coefficient determined from dry weight determinations. The spectral data in Figure 1 show an isosbestic point at 325 nm, which reflects that the level of flavin semiquinone during the reductive titration is quite low.

To assess whether or not the presence of the mediator-titrant dyes [methyl viologen and $K_4Fe(CN)_6$] influenced the value of the determined redox potential, the redox potential of 8α-

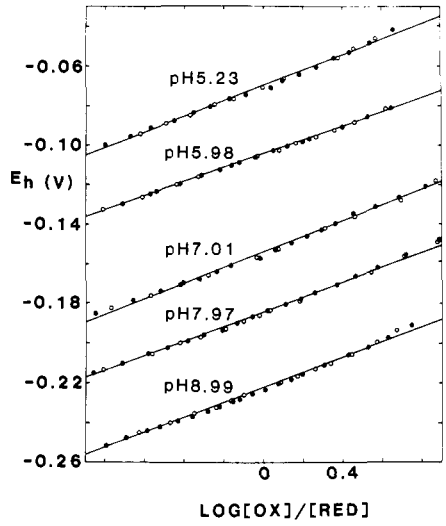

FIGURE 2: Representative Nernst plots from oxidation–reduction titrations of 8α-*N*-imidazolylriboflavin. The best fit (—) to the experimental points, obtained furing reduction (●) and reoxidation (○), was found by linear least-squares regression.

Table II: Concentration Dependence of the Semiquinone Formation Constant, *K*, for 8α-*N*-Imidazolylriboflavin at pH 6.84

flavin concn (μM)	E_m (mV)	*K*	no. of data points
16.6	−142.2 ± 0.4	0.055 ± 0.026	31
36.0	−140.4 ± 0.3	0.041 ± 0.021	20
70.2	−140.6 ± 0.1	0.069 ± 0.007	35
148	−139.5 ± 0.3	0.097 ± 0.019	46

N-imidazolylriboflavin was determined at pH 7.01 by the spectrocoulometric method and, alternatively, with dithionite as the reductant. An E_m value of −154 ± 0.6 mV was determined with dithionite as reductant and an identical value (−154 ± 0.6 mV) determined by the spectrocoulometric method. The semiquinone formation constant (*K*) was found to be 0.36 ± 0.08 with the dithionite method and 0.27 ± 0.08 for the spectrocoulometric method. For all practical purposes, the values are identical and within experimental error.

The data in Table II show that the semiquinone formation constant calculated from the titration data tends to increase as the flavin concentration increases, which is suggestive of significant dimerization (Draper & Ingraham, 1968). To minimize this complication, all redox titrations reported here were performed with flavin concentrations of 40 μM or less.

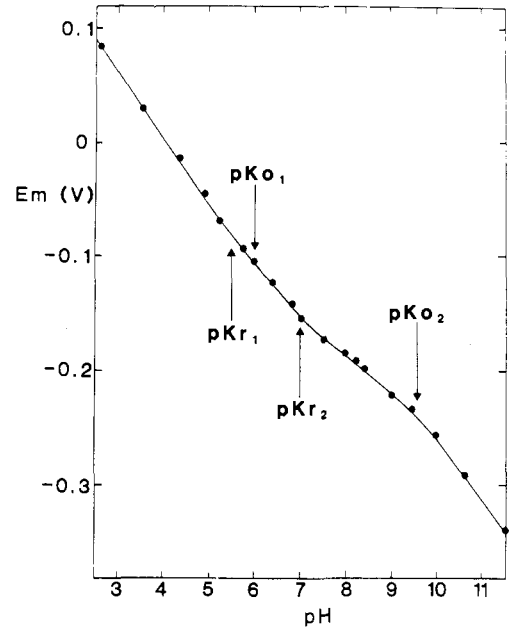

FIGURE 3: pH dependence of E_m for 8α-*N*-imidazolylriboflavin. A computer-simulated E_m vs. pH curve was constructed from the pK_a values shown in Table V. E_m values were obtained from redox titrations as described under Experimental Procedures.

pH Dependence of E_m for 8α-N-Imidazolylriboflavin and Its N-Methylimidazole Analogue. The pH dependence of the oxidation–reduction potential of 8α-*N*-imidazolylriboflavin was determined in the pH range of 2.63–11.53. Figure 2 illustrates representative Nernst plots of the redox data at several selected pH values. Good agreement is observed between the reductive and oxidative phases of the titrations, showing complete reversibility of the system.

The pH dependence of E_m of 8α-*N*-imidazolylriboflavin is shown in Figure 3 and tabulated in Table III. pK_a values of the oxidized and reduced forms of the flavin were obtained by fitting the data to the equation described under Experimental Procedures. The best fit was obtained by using the

Table III: Oxidation–Reduction Potentials for 8α-*N*-Imidazolylriboflavin[a]

pH	E_m (mV)	deviation from theoretical E_m (mV)	*K*	E_1 (mV)	E_2 (mV)	no. of exptl points	buffer system
2.63	84.8 ± 0.7	0.9	0.16 ± 0.08	108	62	24	KP_i
3.54	28.6 ± 0.7	−1.6	0.06 ± 0.02	64	−4	42	KP_i
4.36	−13.9 ± 0.4	3.9	0.10 ± 0.04	15	−43	23	acetate
4.93	−5.4 ± 0.4	4.6	0.23 ± 0.05	−26	−65	27	acetate
5.23	−70.3 ± 0.6	−4.2	0.33 ± 0.08	−56	−84	28	acetate
5.79	−94.3 ± 0.3	−0.1	0.05 ± 0.01	−50	−138	49	acetate
5.98	−104.6 ± 0.2	−1.3	0.12 ± 0.02	−78	−132	30	KP_i
6.40	−123.9 ± 0.7	−0.8	0.12 ± 0.06	−97	−151	28	KP_i
6.84	−142.2 ± 0.4	0.5	0.06 ± 0.03	−105	−179	31	KP_i
7.01	−154.3 ± 0.6	−4.6	0.24 ± 0.08	−136	−173	15	KP_i
7.50	−173.1 ± 0.8	−5.0	0.38 ± 0.12	−161	−186	32	KP_i
7.97	−183.9 ± 0.5	−0.5	0.23 ± 0.07	−165	−203	33	KP_i
8.21	−191.0 ± 0.4	0.3	0.04 ± 0.02	−149	−233	40	Tris-HCl
8.39	−198.2 ± 0.6	−1.2	0.23 ± 0.07	−180	−217	30	Tris-HCl
8.99	−222.0 ± 0.5	−3.3	0.27 ± 0.07	−205	−239	34	Tris-HCl
9.47	−233.1 ± 0.4	1.5	0.05 ± 0.01	−195	−272	42	Tris-HCl
10.01	−255.5 ± 0.6	3.5	0.23 ± 0.08	−237	−274	26	carbonate
10.62	−290.0 ± 0.6	1.4	0.16 ± 0.08	−266	−314	22	carbonate
11.53	−338.5 ± 2.2	5.5	0.20 ± 0.19	−322	−354	17	KP_i

[a] Potentials were calculated from both reduction and reoxidation data. The error in E_m does not include the variability (0.7 mV) in the reference electrode nor the error in pH measurement (±0.02 unit). The theoretical E_m at a given pH was calculated from the pK_a values in Table V.

Table IV: Oxidation–Reduction Potentials for 8α-(*N*-Methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin[a]

pH	E_m (mV)	deviation from theoretical E_m	K	E_1 (mV)	E_2 (mV)	no. of points	buffer system
2.62	124.3 ± 1.2	8.3	0.16 ± 0.1	148	100	16	KP_i
4.22	18.2 ± 0.2	−3.4	0.23 ± 0.03	37	−1	34	acetate
5.23	−44.4 ± 0.4	−9.5	0.22 ± 0.04	−25	−64	41	acetate
5.98	−69.1 ± 0.2	3.5	0.26 ± 0.03	−52	−87	30	KP_i
6.68	−96.0 ± 0.4	3.1	0.12 ± 0.04	−69	−123	28	KP_i
7.29	−116.8 ± 0.4	1.5	0.30 ± 0.06	−132	−118	26	KP_i
7.75	−132.9 ± 0.2	−0.1	0.29 ± 0.03	−116	−150	35	KP_i
8.39	−152.8 ± 0.9	−0.2	0.28 ± 0.10	−137	−169	27	Tris-HCl
8.99	−171.0 ± 0.7	1.9	0.45 ± 0.13	−161	−181	28	Tris-HCl
9.49	−195.3 ± 1.1	−2.3	0.75 ± 0.27	−191	−200	28	carbonate
10.01	−220.3 ± 1.7	−2.2	0.87 ± 0.40	−217	−224	26	carbonate

[a] The oxidation–reduction potentials, errors, and theoretical values were calculated as described in the legent to Table III and under Experimental Procedures.

values of $pK_{o1} = 6.0$ and $pK_{o2} = 9.7$ as determined above and finding the values of pK_{r1} and pK_{r2} that gave the least deviation from the experimental E_m values. This was accomplished by using a computer-simulated E_m vs. pH plot and taking a standard deviation of the sum of the E_m errors. From this approach, a pK_{r1} value of 5.5 and a pK_{r2} value of 6.9 were found to give the best fit of the experimental data points. A maximum deviation of 5.5 mV from the theoretical E_m values was found (Table III), and the standard deviation of the sum of the errors was found to be ±3.0 mV. This uncertainty lends to an uncertainty of the pK_a values of less than 0.1 pH unit.

The two pK_r values could be assigned to either the imidazole ionization (VIII to IX, Scheme I) or to N(1) ionization of the dihydroisoalloxazine ring (VI to VIII, Scheme I). To distinguish between these possibilities, the pH dependence of the oxidation–reduction potential of 8α-*N*-methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin was determined. In the case of this flavin analogue, the alkylated imidazole ring has a positive charge throughout the pH range tested, and the only observable pK_r should be that due to the ionization of the N(1) position of the reduced flavin. Table IV lists the E_m values (and standard errors) at the pH values tested, and the data are plotted relative to a theoretical E_m vs. pH plot in Figure 4 in which pK_r was assigned to be 5.9 and pK_o [corresponding to N(3) flavin ionization] to be 9.5. The fit of the experimental data relative to the theoretical showed a standard error to ±4.6 mV, which is equivalent to a maximum error in the respective pK_a values of ±0.15 pH unit. From these data, we assign the pK_{r1} of 5.5, observed for 8α-*N*-imidazolylriboflavin hydroquinone, to the N(1) ionization of the flavin ring. Consequently, pK_{r2} (6.9) is assigned to the ionization of the imidazole ring of the flavin hydroquinone. That this assignment is correct is also shown by ^1H NMR spectroscopy of the reduced flavin (Williamson & Edmondson, 1985).

These data show that the pK_a of the imidazole ring increases by approximately 1 pH unit on reduction of the flavin, whereas the presence of an 8α-imidazole substituent decreases the pK_a of the N(1) position of the reduced flavin ring by 0.8 pH unit.

Effect of pH on E_1 and E_2 for 8α-N-Imidazolylriboflavin and Its N-Methylimidazole Analogue. As was done earlier for riboflavin and FMN (Draper & Ingraham, 1968), the redox data were subjected to iterative computer analysis in order to determine values for the semiquinone formation constant (K) and subsequently the values for E_2 and E_1 as a function of pH. In principle, this approach should provide estimates of the pK_a values for imidazole ionization and N^5-isoalloxazine ionization for the semiquinone form of the flavin. The value of K is extremely sensitive to minor experimental uncertainties when $K < 1$ (Draper & Ingraham, 1968). For example, if $K \sim 0.1$, an uncertainty of 0.3 mV

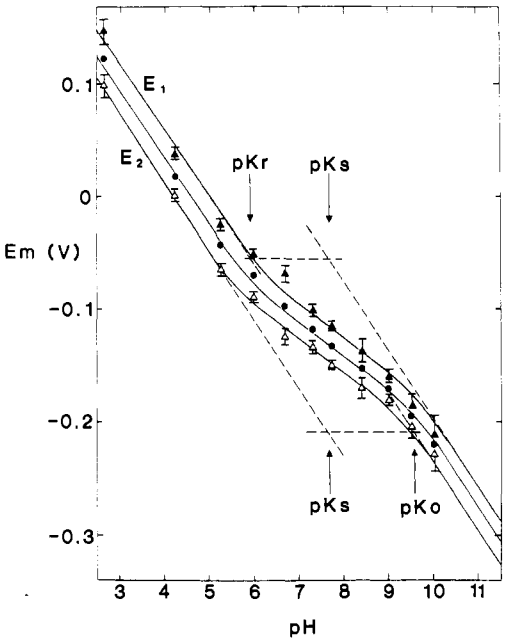

FIGURE 4: pH dependence of E_m, E_1, and E_2 for 8α-(*N*-methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin. The central solid line is the computer-simulated curve for $pK_o = 9.5$ and $pK_r = 6.9$, fitted to the experimentally obtained E_m values (●). E_1 (▲) and E_2 (▲) were used to estimate pK_s (7.6) as described in the text. Titrations above pH 10 showed complications due to hydrolysis of the ribityl tetra-*O*-acetyl groups.

in the index potential of the Nernst plot leads to a 50% error in the value of K. When the value of K is smaller, the errors became larger and the standard deviations in E_1 and E_2 increase exponentially.

The calculation of pK_{s1} and pK_{s2} was aided by the more accurately determined pK_a values for the oxidized and reduced forms of the flavin. Figure 5A was constructed by the procedure of Draper & Ingraham (1968) and of Clark (1960) in the following manner. When a slope (ΔE_m/pH) changes to a lower value with increasing pH, the inflection point corresponds to the pK_a of the most reduced species of the couple. Conversely, a shift in slope to a higher value corresponds to the pK_a of the most oxidized species of the couple. For a one-electron process, the only allowed slopes are 0, 60, 120, etc. (mV/pH), which correspond to proton transfers of 0, 1, 2, etc., respectively. The slopes at the pH extremes for both E_1 and E_2 are 60 mV/pH and parallel that of the E_m plot. The E_2 plot was altered in slope by 60 mV/pH_a at the two pK_o values determined above while the E_1 plot was altered in slope in a similar fashion at pK_{r1} (Figure 5). The other known factor is the change in slope of E_1 at pK_{r2}, which was deter-

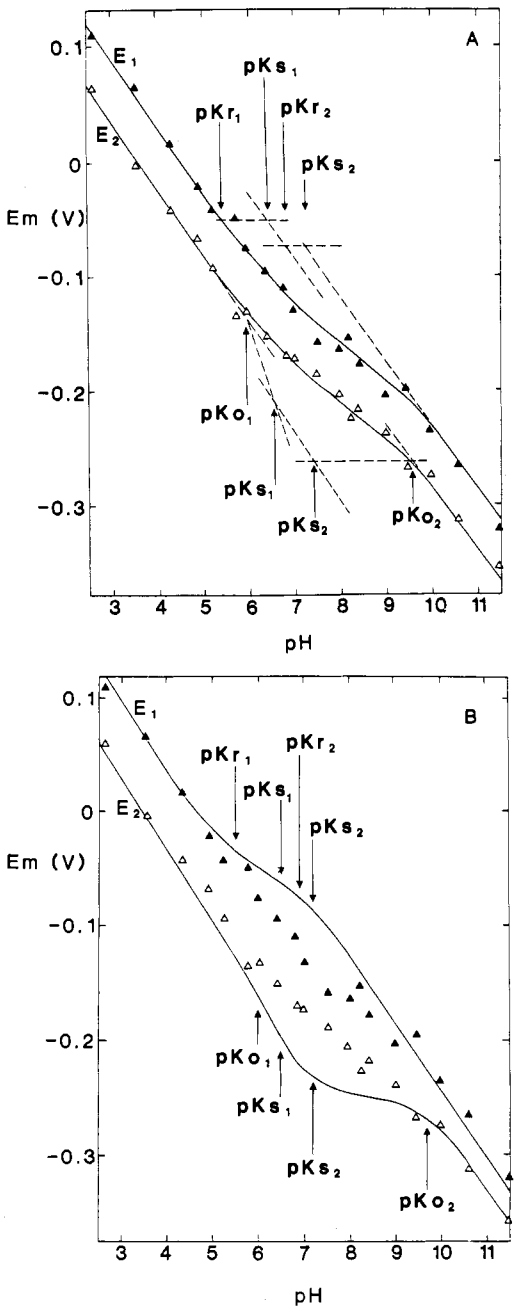

FIGURE 5: (A) pH dependence of E_1 and E_2 for 8α-*N*-imidazolylriboflavin. E_1 (▲) and E_2 (△) were calculated from the Michaelis equation. Graphical representation of how the pK_a values in Table V were estimated by the method of Draper & Ingraham (1968) as explained in the text. (B) Comparison of computer-simulated E_1 and E_2 values vs. pH of the pK_a values obtained in (A) with the E_1 and E_2 values determined by iterative analysis of the experimental redox data as described under Experimental Procedures.

mined to be 6.9 from the E_m plot. Finally, the best values for pK_{s1} and pK_{s2} were estimated by placing the remaining guidelines such that both pK inflections coincided for both E_1 and E_2 plots. This defines the relative difference between pK_{s1} and pK_{s2} but not their absolute values. Previous work (Edmondson et al., 1977) estimated the pK_a for N(5) ionization of the 8α-N^1- and 8α-N^3-histidylriboflavin analogues to be 7.2 as judged from the pH dependence of the reactivity of the semiquinone form with O_2. By use of this value for pK_{s2}, the value of pk_{s1} was estimated to be 6.5, which would be assigned to the ionization of the imidazole residue.

The calculated E_1 and E_2 values in Figure 5A deviate markedly from those theoretically predicted in the pH 6–9 region (Figure 5B) with the equation given under Experimental procedures. Although this discrepancy does not influence the pK estimates drawn from Figure 5A, it is of interest to note that a similar discrepancy is apparent in the case of riboflavin or FMN in the same pH region (Draper & Ingraham, 1968). The deviation from the theoretical behavior is due to the inherent error of E_1 and E_2 from the uncertainty in the calculation of K. Indeed, Michaelis et al. (1937) reported that their E_i value was between 14.8 and 17.0 mV for riboflavin and some of its derivatives over a wide pH range (3–11). These authors remarked that "the scattering of the individual results around the average value (E_i or K) is evenly distributed among flavins throughout this pH range." "Therefore, we believe that E_i (and hence K) is the same, within the limits of error, throughout the pH range." This observation appears to have withstood the test of time as judged by the values of K for riboflavin and FMN (Draper & Ingraham, 1968) and by the results presented here.

The pK_a for flavin N(5) ionization of 8α-(*N*-methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin was determined to be 7.6 from analysis of E_m, E_1, and E_2 curves (Figure 4, Table IV). This value is in reasonable agreement with the value of 7.2 determined for 8α-(N^3-N^α-acetyl-N^1-methylhistidyl)tetra-*O*-riboflavin by following the pH dependence of the rate of semiquinone reaction with O_2 (Edmondson et al., 1977).

Discussion

The results of this study demonstrate that the pK_a of the imidazole moiety of 8α-imidazole-substituted flavins is dependent on the oxidation level of the flavin. In the oxidized flavin, the pK of the imidazole ring is 6.0, which is quite similar to the pK_a observed for *N*-benzylimidazole (Grimmett, 1980). Comparison of the pK_a of 8α-*N*-imidazolylriboflavin with those reported for 8α-N^1- and 8α-N^3-histidylriboflavin (which are 4.7 and 5.2, respectively) (Edmondson et al., 1976) demonstrates that the histidyl side chain and amino and carboxyl groups perturb the pK_a of the unsubstituted imidazole nitrogen. Recent studies (Boschcov et al., 1983) have suggested the α-amino group to exert an inductive effect, suggestive of an ion–dipole interaction between the protonated amino group and the unprotonated imidazole ring. Whether such a mechanism occurs in the 8α-*N*-histidylflavins remains to be determined. These complicating factors were the prime reason for using an 8α-*N*-imidazole substituent in this study rather than 8α-*N*-histidyl-substituted flavins.

On reduction of the flavin ring, the pK_a of the 8α-imidazole ring is increased by approximately 1 pH unit to a value close to that the free imidazole (pK_a = 7–7.2) or of 1-methylimidazole (pK_a = 7.3) (Grimmett, 1980). On the basis of ^{1}H NMR studies (Williamson & Edmondson, 1985), this pK_a reflects an intrinsic value rather than an apparent pK_a. This demonstrates that the electron-withdrawing inductive effect of the oxidized isoalloxazine ring has been abolished and is compensated for in the opposite direction (i.e., there is an electron-donating inductive effect) of flavin reduction. Thus, the ionization behavior of the 8α-imidazole ring is more like a 1-methylimidazole analogue rather than an *N*-benzylimidazole. Our estimate for the pK_a of the 8α-imidazole ring in the semiquinone (pK_a = ~6.5) is intermediate between the pK_a values for the oxidized and reduced forms of the flavin and is reasonable in that the electron-withdrawing or -donating activity of the flavin semiquinone lies somewhere between that of the oxidized and reduced forms.

Perturbations of pK_a of the ionizable positions of the flavin ring are observed in the three oxidation–reduction levels due

Table V: Summary of pK_a Values and Oxidation–Reduction Potentials for Various Flavins

flavin	flavoquinone		semiquinone		hydroquinone		
	pK_{o1} (imid)	pK_{o2} [Fl N(3)]	pK_{s1} (imid)	pK_{s2} [Fl N(5)]	pK_{r1} (imid)	pK_{r2} [Fl N(1)]	E_m (mV), pH 7.0
8α-*N*-imidazolylriboflavin	6.0	9.7	6.5	7.2	5.5	7.0	−154
8α-(*N*-methyl-*N*-imidazolium)tetra-*O*-acetylriboflavin		9.5		7.6		5.9	−108
8α-*N*-imidazolyltetra-*O*-acetylriboflavin	6.0	ND[c]	ND	ND	ND	ND	−112
riboflavin[a]		10.0		8.3		6.3	−200
FAD[b]		10.5				6.7	−209
FMN[a]		10.4		8.6		6.7	−205

[a] From Draper & Ingraham (1968). [b] From Lowe & Clark (1956). [c] Not determined.

to the 8α-imidazole substituent. No major alterations are observed on methylation of the imidazole to form the positively charged 8α-imidazolium form. A substantial effect on the measured $E_{m,7}$ values is observed on comparison of the riboflavin and tetra-*O*-acetyl forms (Table V), suggesting appreciable side chain–isoalloxazine ring interaction. The pH dependence of E_m could be easily done with the (*N*-methyl-*N*-imidazolium)tetra-*O*-acetylflavin analogue but not with imidazolyltetra-*O*-acetylriboflavin. A slow but significant rate of hydrolysis of the side-chain acetyl groups occurred during the titrations, which is pH-dependent and apparently involves catalysis by the imidazole substituent. Due to the large change in potential between the acetylated and deacetylated forms, an appreciable hysteresis is observed on comparing the oxidative and reductive titrations. Thus, the pH-dependent redox studies could only be done on the 8α-flavin at the riboflavin level.

Of greater significance to those flavoenzymes containing 8α-histidyl-substituted flavins is the result that any pH dependence of oxidation–reduction potential studies must take into account the ionization of the 8α-histidyl ring as well as the ionic properties of the semiquinone and hydroquinone forms of the isoalloxazine ring. Therefore, the apparent pK_a of 8.0 observed for the 8α-N^3-histidyl-FAD of succinate dehydrogenase (Ohnishi et al., 1981) is more likely due to the ionization of the 8α-imidazole substituent rather than the flavin semiquinone N(5) ionization, inasmuch as no alterations in ESR linewidth or sensitivity of the ESR linewidth to D_2O is observed in the pH range expected for anionic to neutral flavin semiquinone transformation (Edmondson et al., 1981).

Recent electrochemical studies on *Schizophyllum commune* cholesterol oxidase (Ikeda et al., 1981), a flavoenzyme containing a covalently bound 8α-N^1-histidyl-FAD (Kenney et al. 1979), have demonstrated pK_a values of 6.4 (pK_r) and 8.5 (pK_o), which differ considerably from those observed with free FAD (pK_r ~7 and pK_o ~10). Although the absolute values of these pK_a values may be influenced by absorption to the mercury electrode surface and further work is required to assign these observed pK_a values to discrete components of the covalently bound flavin, it is tempting to speculate that the pK_o may reflect 8α-imidazole ionization of the oxidized enzyme while pK_r reflects N(1) ionization of the reduced flavin. Further work is required to test whether this speculation is, indeed, correct.

The demonstration of a redox-linked ionization of the 8α-imidazole substituent suggests a role of this substituent in modulating the redox properties of the catalytically involved flavin coenzyme in those enzymes containing covalently bound 8α-histidyl flavins. Although other noncovalent protein–flavin interactions are undoubtedly involved in determination of the redox potential, the results of this study would suggest that modulation of the imidazole pK_a of either the oxidized or reduced forms of the enzyme by other amino acid residues or by substrate binding could be an effective means of modulating the flavin redox potential. This could be one of the reasons for the existence of 8α-imidazole-substituted flavins in certain flavoenzymes.

It remains for future work to test the validity of this hypothesis. As an approach to this question, a careful pH-dependent redox potential study of flavoenzymes containing 8α-histidylflavins is certainly in order. Efforts along these lines are currently under way in this laboratory.

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Purification and Characterization of Two β-1,4-Endoglucanases from *Thermomonospora fusca*†

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ABSTRACT: Two β-1,4-endoglucanases were isolated in nearly pure form from the culture supernatant of *Thermomonospora fusca* strain YX. The purification procedures included gel chromatography, ion-exchange chromatography, hydroxylapatite chromatography, and preparative gel electrophoresis. These enzymes and their proteolytic breakdown products account for at least 90% of the total extracellular carboxymethylcellulase activity, although at least one other β-1,4-endoglucanase is present. Even though these enzymes are essential for the hydrolysis of filter paper by the extracellular fraction, they are not sufficient. The specific activity of the starting material on filter paper was 4 times that of the most active purified enzyme. Even though they are both endoglucanases, these two enzymes differ in their molecular weights, substrate specificities, and carbohydrate contents and are immunologically distinct proteins. The most active enzyme (E_1) has a specific activity greater than 700 IU/mg on carboxymethylcellulose (CMC) when assayed at 55 °C in 0.05 M potassium phosphate buffer, pH 6.5. Its molecular weight is 94 000 determined by both sodium dodecyl sulfate gel electrophoresis and glycerol gradient centrifugation, showing that it is a monomeric enzyme. It contains less than 1% carbohydrate and has an isoelectric point at pH 3.5. Its temperature optimum is 74 °C, and it has a broad pH optimum centered at pH 6. The products of cellulose hydrolysis catalyzed by E_1 are mainly cellobiose with a small amount of glucose. Its K_m for CMC is 360 μg/mL; it is stimulated by Ca^{2+} or Mg^{2+} and totally inhibited by Hg^{2+}. The other enzyme (E_2) has a specific activity of 77 IU/mg on CMC. Its molecular weight is 46 000, and it is also a monomeric enzyme. It is a glycoprotein containing 25% carbohydrate by weight and has an isoelectric point at pH 4.5. Its temperature optimum is 58 °C, and it has a broad pH optimum centered at pH 6. The products of cellulose hydrolysis catalyzed by E_2 are glucose, cellobiose, cellotriose, and higher oligomers. Its K_m for CMC is 120 μg/mL, and it is slightly inhibited by Ca^{2+} or Mg^{2+} and greatly inhibited by Hg^{2+}. Neither enzyme is inhibited appreciably by 10% glucose, but both are about 50% inhibited by 10% cellobiose.

The study of cellulases is of practical importance because of their potential role in the conversion of renewable biomass into chemicals or fuel. Cellulases are also interesting because cooperative interactions between two or more enzymes are required for the optimal degradation of crystalline cellulose. While considerable progress has been made in the characterization of the cellulases produced by the mesophilic fungi *Trichoderma* (Bissett, 1979; Farkas et al., 1982; Shoemaker & Brown, 1978; Weber et al., 1980) and *Sporotrichum pulverulentum* (Erickson & Pettersson, 1978a,b) and the anerobic bacterium *Chlostridum thermocellum* (Ng & Zeikus, 1981b, Petre et al., 1981), much less is known about cellulases produced by thermophilic aerobic bacteria.

The potential advantages of thermophilic enzymes in practical applications center around their great stability. In this work the organism *Thermomonospora fusca* was chosen because it is a thermophilic, aerobic, bacterium that produces a very active cellulase. There have been a number of studies of the crude cellulase produced by different *Thermomonospora* species (Moreira et al., 1981; Hägerdal et al., 1978a,b), but the detailed characterization of purified enzymes has not been reported. This paper describes the purification and characterization of the two most active *T. fusca* β-1,4-endoglucanases.

Materials and Methods

Organism. *Thermomonospora fusca* strain YX was isolated by W. D. Bellamy, Cornell University. This organism has been shown by M. P. Lechevalier to be a thermophilic, filamentous actinomycete. The bacterium contains type III cell wall, forms aerial mycelium, and reacts variably with the Gram stain.

Crude Enzyme Preparation. A 48-h, 200-mL shake culture of *T. fusca* YX was used to inoculate a 14-L fermentor (New Brunswick Scientific Corp., New Brunswick, NJ) containing

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