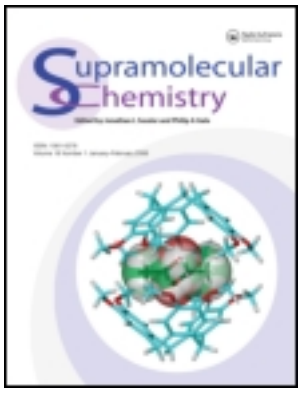

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Redox reaction between *m*-thiocresol and riboflavin glycosides with 2:1 complex formation; regulation by the steric effect of sugar in the side chain

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We investigated the reduction of riboflavin-2′, 3′, 4′, 5′-tetra-acetate (AcB_2), riboflavin-1′-glucoside-2″, 3″, 4″, 6″, 2′, 3′, 4′, 5′-hepta-acetate (AcB_2gl) and lumiflavin using *m*-thiocresol (mTc) in the presence of tetrabutylammonium hydroxide. The series of rate constants for AcB_2 and AcB_2gl reductions indicated that modified Lineweaver–Burk plots were best fit by assuming a 1:2 complex formation. The complex formation in the reaction was supported by the 2-D nuclear Overhauser enhancement spectroscopy and circular dichroism spectra. The modified Michaelis–Menten constants (K_m) for AcB_2 and AcB_2gl with mTc were 1.32 and $0.86 \times 10^{-3}\,M^2$, respectively, and the maximum rate constant k_2 were 4.45 and $4.35 \times 10^{-2}\,M^{-1}\,s^{-1}$, respectively. The $E_{1/2}$ values of AcB_2 and AcB_2gl were − 331 and − 341 mV, respectively, which indicated that their reduction activities were almost the same. It was established that the redox function depended on the formation of the complex and was regulated by the steric effect of the sugar in the side chain.

Keywords: riboflavin glycoside; *m*-thiocresol; Lineweaver–Burk plot; steric effect; host–guest complex

Flavoproteins play a key role in oxidation–reduction systems catalysing the electron transfer (*1*). The functions of flavoproteins may be modified artificially so that they associate reversibly by providing an appropriate self-organising system for flavin and the other electron acceptors. Riboflavin (B_2), 7,8-dimethyl-10-((2*R*,3*R*,4*S*)-2,3,4,5-tetrahydroxyphenyl)benzo-[*g*]pteridine-2,4(3*H*,10*H*)-dione, which is known as vitamin B_2, is the central component of flavoproteins. B_2 is generally stable during heating, but is sensitive to light, oxygen and alkaline solution. The derivatives of 7,8-dimethyl-iso-alloxazine that possess different functional groups in the side chain at position 10 are defined as flavins. In biochemical reactions, flavins undergo reversible redox conversion atoms N(5) and N(1). Many model systems for flavin catalyst reactions have been reported (*2*).

Glycosides are molecules in which a sugar is bound to a non-carbohydrate group; they play an important role in living organisms. Many plants store chemical molecules in the form of inactive glycosides, which can be activated by hydrolysis using enzymes. The bioavailability of glycoside usually depends on the type of glycosidic bond and glycone. It has also been known that riboflavin α-glycoside (B_2gl) is a metabolite; however, its metabolic pathway and the role of the glycoside have not yet been revealed (*3*). B_2gl exhibits high water solubility, but it is still sensitive to light and oxygen. By photo-irradiation, B_2gl is resolved into B_2 and glucose, and then B_2 is resolved to lumiflavin (LF) and lumichrome. Acetylated B_2gl is stable during photo-irradiation. To elucidate the redox-reaction mechanism between riboflavin derivatives, kinetic studies were carried out using acetylated flavin and *m*-thiocresol (mTc) in acetonitrile with tetrabutylammonium hydroxide (TBAH), by means of UV spectra. In this paper, we report the preparation of acetylated B_2 derivatives, and discuss the kinetic parameters of their reduction using mTc which induces a 1:2 complex formation.

Riboflavin-1′-glucoside (B_2gl) was prepared using riboflavin and maltose with an enzyme prepared from *Aspergillus oryzae* extract in a citrate buffer (pH 3.5) at 30°C for 48 h, as shown in Figure 1 (*4*). The separation and purification of B_2gl was performed using HP-20. B_2gl was obtained in yields of 48% and Rf value of 0.21 (ethylacetate/pyridine/aq.:18/6/1). Riboflavin-2′,3′,4′,5′-tetra-acetate (AcB_2) was prepared by the reaction of riboflavin with acetic anhydride in pyridine according to a previous report (*5*). After removing pyridine, crude AcB_2 was recrystallised from a mixture of ethanol and chloroform (12.7% yield). The identification of AcB_2 was

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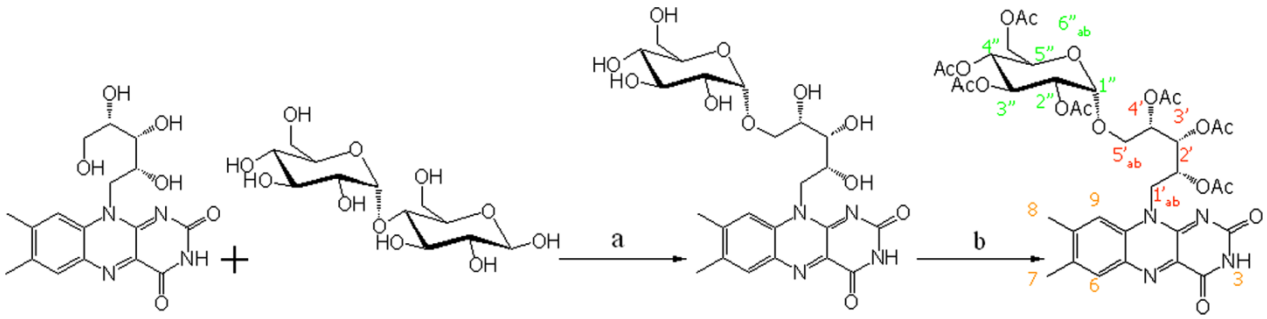

Figure 1. Preparation of riboflavin glycoside and acetylation.

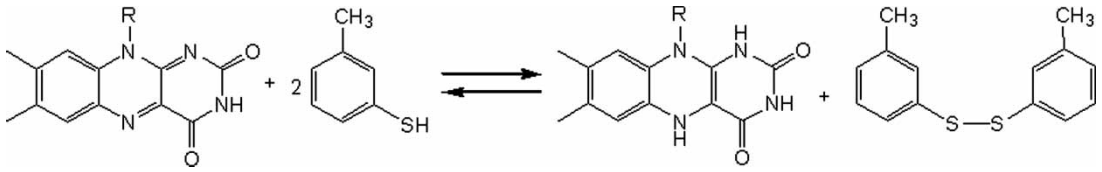

Figure 2. Redox reaction between flavin and mTc.

carried out by TLC, NMR and ESI-MS spectroscopy. Rf value: 0.21 (hexane/ethylacetate = 1:3), ESI-MS *m/z* calcd for $C_{25}H_{28}N_4O_{10}$: 567.1698; found 567.1654. ^{1}H NMR (500 MHz, CD_3CN, 60°C, ppm): δ 1.73, 2.04, 2.18, 2.24 (s 3H acetyl methyl), δ 2.42 (s 3H methyl 8a), δ 2.54 (s 3H methyl 7a), δ 4.20 (dd 1H ribityl 4′), δ 4.37(dd 1H ribityl 3′), δ 4.90 (brd 1H ribityl 5′a), δ 5.10 (brd 1H ribityl 5′b), δ 5.29 (m 1H ribityl 2′), δ 5.40 (m 1H ribityl 1′b), δ 5.65 (m 1H ribityl 1′a), δ 7.53 (s 1H phenyl 6), δ 8.00 (s 1H phenyl 9), δ 8.34 (s 1H imide 3). Riboflavin-1′-glucoside-2″, 3″, 4″, 6″, 2′, 3′, 4′, 5′-hepta-acetate (AcB_2gl) was also obtained from riboflavin-1′-glucoside by applying the procedure described above. Rf value: 0.16 (hexane/ethylacetate = 1:3), ESI-MS *m/z* calcd for $C_{37}H_{43}N_4O_{18}$: 855.2542; found 855.2567. ^{1}H NMR (500 MHz, CD_3CN, 60°C, ppm): δ 1.65, 1.91, 1.95, 2.00, 2.15, 2.19 (s 3H acetyl methyl), δ 2.37 (s 3H methyl 8a), δ 2.49 (s 3H methyl 7a), δ 3.73 (dd 1H ribityl 4′), δ 3.82 (dd 1H ribityl 3′), δ 3.92 (qq 1H glucose 5″), δ 4.04 (dd 1H glucose 6″b), δ 4.14 (dd 1H glucose 6″a), δ 4.77 (dd 1H glucose 2″), δ 4.86 (brd 2H ribityl 5′ab), δ 4.95 (t 1H glucose 4″), δ 5.06 (d 1H glucose 1″), δ 5.25 (m 1H ribityl 2′), δ 5.35 (t 1H glucose 3″), δ 5.39 (m 1H ribityl 1′b), δ 5.57 (m 1H ribityl 1′a), δ 7.38 (s 1H phenyl 6), δ 7.95 (s 1H phenyl 9), δ 8.25 (s 1H imide 3).

We studied the reduction of riboflavin to 1,5-dihydroriboflavin in acetonitrile at 293 K (Figure 2). The reaction was nearly quantitative, as determined by spectroscopy when TBAH and an excess of mTc (300-fold) was used. The rate of reduction was measured by following the characteristic absorption of flavin (F) at λ_{max} 442 nm and the fluorescence emission intensity at λ_{max} 505 nm. The products, FH_2 and di-(*m*-methyl)phenyl-disulphide were identified by comparing the ^{1}H NMR spectra with the reported spectrum of FH_2 and di-(*m*-methyl) phenyl-disulphide (*5b*). Good pseudo-first-order rate data were obtained. The value of the observed rate constant k_{obs} for the reduction of AcB_2 (1.4×10^{-4} M) with mTc (4.2×10^{-2} M) in the presence of TBAH (3.3×10^{-3} M) is comparable to a previously reported value (*5b*). The k_{obs} of acetylated B_2 flavins indicates three times greater than that of LF. k_{obs} decreases at high temperatures (Table 1). The reduction reaction was analysed according to the standard and modified Michaelis–Menten scheme (Equations (1a), (1b) and

Table 1. Observed pseudo-first-order rate constants for the reduction in acetylated B_2 derivatives with *m*-thiocresol.

	$k_{obs}(10^2\ s^{-1})$		
Flavin	293 K	323 K	343 K
Lumiflavin (LF)	0.9 0.8[a]	0.62	0.55
AcB_2	2.67 2.30[a] 2.59[b]	1.77	1.58
AcB_2gl	2.97 3.16[a]	1.92	1.60

[a] Measured by the fluorescence emission intensity at λ_{max} 505 nm.
[b] 298 K, ref. (*5b*).

Table 2. Kinetic parameters, Michaelis–Menten constants (dissociation constants) and rate constants for the reduction obtained by modified Lineweaver–Burk plots (Equation (2c)).

Flavin	k_2 (10^2 s^{-1})	K_m(10^3 M^2)	k_2/K_m
AcB_2	4.46	1.32	3.37
AcB_2gl	4.33	0.86	5.00

Table 3. $E_{1/2}$ values of riboflavin derivatives.

	$E_{1/2}$(mV)		
Flavin	293 K	323 K	343 K
AcB_2	−331	−341	n.d.
AcB_2gl	−314	−338	−392
B_2gl	−446	−464	−480

(2a), (2b), respectively).

$$F + S \rightleftarrows F{\cdot}S \rightarrow P, \tag{1a}$$

where S is the electron receptor and P is the product.

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{(k_{-1} + k_2)}{k_1 k_2}[S]_0, \tag{1b}$$

$$F + 2S \rightleftarrows F{\cdot}S \rightarrow P, \tag{2a}$$

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{(k_{-1} + k_2)}{k_1 k_2}[S]_0^2, \tag{2b}$$

$$K_m = \frac{(k_{-1} + k_2)}{k_1}, \tag{3}$$

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{K_m}{k_2}[S]_0, \tag{1c}$$

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{K_m}{k_2}[S]_0^2. \tag{2c}$$

The correlation coefficients of the plots according to (1c) for AcB_2 and AcB_2gl are 0.9514 and 0.958, respectively. The coefficients according to (2c) for AcB_2 and AcB_2gl are 0.9912 and 0.9976, respectively. The redox reactions proceed through a complex formation of one acetylated B_2 derivative and two mTc molecules. The rate constants k_2 and K_m for the reaction of totally complexed FS_2 were evaluated from (2c); the resultant kinetic parameters are given in Table 2. The maximum rate constant k_2 for AcB_2gl is almost the same as that for AcB_2. Cyclic voltammetry is generally used to study the electrochemical properties in solution (*6*).This result is consistent with that obtained by cyclic voltammetry (Table 3). The K_m value for AcB_2gl is considerably smaller than that for AcB_2. This indicates that in AcB_2gl, the mTc is embedded strongly in the cleft surrounded by the acetyl glucose group and alloxazine group, resulting in enhanced binding, whereas the cleft in AcB_2 is not deep enough to form a stable complex. AcB_2 and AcB_2gl showed induced circular dichroism (CD) spectra peaking at 440, 340 and 255 nm. The result indicated that the sugar groups of AcB_2 (ribityl group) and AcB_2gl (ribityl and glucose groups) stay close to the 7,8-dimethyl-iso-alloxazine group. A direct evidence for the relative orientation of the mTc and flavin has been obtained by the 2-D NMR NOESY method (Figure 3). The cross peaks between the 5′ab protons and the protons of mTc and between the 5′ab protons and 6 proton were observed. These situations lead to an apparent overall increase in the reduction rate k_2/K_m for AcB_2gl; this rate is approximately 1.5 times greater than that for AcB_2. The phenomenon ‘supramolecular regulation’, a redox reaction, is regulated only by the steric effect of sugar. Only methyl group is substituted at position 10 in LF; a straight line in both of the plots according to (1c) and (2c) could not be observed. No induced CD was observed. The result is an example of how a catalytically inactive group can regulate the catalytic activity. Sugar groups

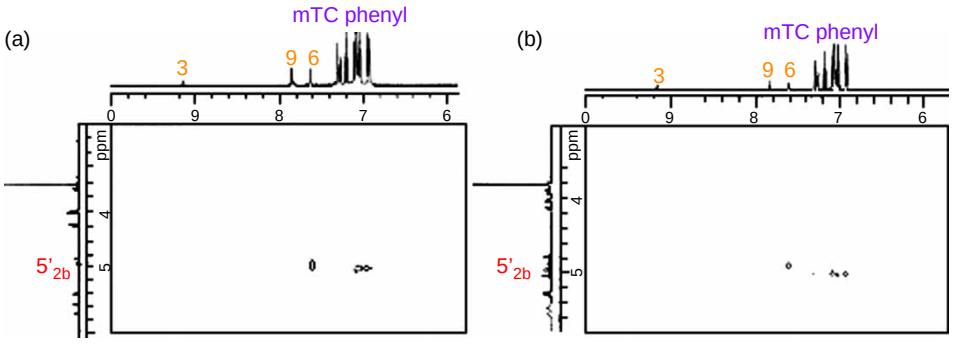

Figure 3. NOESY spectra of AcB_2 (a) and AcB_2gl (b) in CD_3CN at 293 K.

had attracted attention as the sensing group for molecular recognition; however, now they can also be used as 'conformational control factors'. The preparation of various glycosides and a detailed analysis of their molecular structure are under investigation.

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