Effects of phosphate on the binding of FMN and riboflavin by apoflavodoxin from *Desulfovibrio vulgaris* (Hildenborough).

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Flavodoxins are small flavoproteins which contain a single molecule of FMN as prosthetic group. They have been isolated from a variety of microorganisms where they function as electron-transfer proteins at low redox potential [1,2]. The small size of flavodoxins and the ease with which their apoproteins can be prepared makes them suitable models for studies on the kinetics and thermodynamics of interaction between protein and flavin. The protein can be reversibly separated from FMN by precipitation with trichloroacetic acid [3]. Some apoflavodoxins bind only FMN and flavins that carry the terminal phosphate of FMN [4], while others, such as that from *Desulfovibrio vulgaris*, are less specific since they also bind riboflavin [5]. It has been shown that the rates of association of apoflavodoxins and flavins vary with pH and the ionic composition of the medium [4-8].

In this report on the effect of phosphate on the binding of flavins by recombinant apoflavodoxin from *D. vulgaris* [9,10], dissociation constants were determined by fluorescence titration [3] and rate constants for binding were obtained under pseudo-first order conditions using a stopped-flow spectrofluorimeter.

The binding of FMN to apoflavodoxin was studied in the pH range 5-8 using monoanionic buffers (Tris-HCl, sodium cacodylate or sodium acetate, all at 20 mM) and varying the ionic strength with NaCl, and also in sodium phosphate buffer (pH 6-7.5) of varying concentration. The values obtained for the second order rate constant varied with the composition of the buffer and with pH (Fig. 1). The rate of binding increased with ionic strength when NaCl was used, approaching a maximum at ionic strengths greater than about 400 mM. With phosphate the rate increased at low concentrations but then decreased as the phosphate concentration was increased further. This decrease was more marked at higher pH, suggesting that the effect was caused by dianionic phosphate ($pK_a = 7.2$). In support of this, it was found that phosphate had a similar effect to NaCl when it was used to increase the ionic strength in acetate buffer at pH 5. Sulphate anion at pH 7 gave intermediate values for the rate constants (Fig. 1), an effect that might be due either to the dianionic nature of this ion or to a structural similarity to HPO_4^{2-} . The effects of SO_3^{2-} could not be investigated because of a specific interaction of sulphite with flavins [11].

Dissociation constants measured at pH 7 in phosphate or Tris buffer at the same ionic strengths were similar ($2.08 \pm 0.31 \times 10^{-10}$ M in 100 mM phosphate; $1.53 \pm 0.48 \times 10^{-10}$ M in 20 mM Tris-HCl with 100 mM NaCl), indicating that the complex, though more slowly formed in the presence of phosphate, also dissociates more slowly.

In contrast to the above results with FMN, phosphate increased the rate of binding of riboflavin by the apoprotein by a factor of 10 and decreased the dissociation constant of the riboflavin complex by a similar amount. Sulphate at very high concentrations had a similar effect, while chloride had very little effect over the concentration range tested. These results suggest that phosphate, or some other dianion, is required for rapid and tight binding of riboflavin. It is possible that phosphate ion binds at the same site as the phosphate group of FMN and induces a protein conformational change that allows riboflavin to bind.

Fig. 1. Effect of buffer composition on the second order constant of binding of FMN to apoflavodoxin. FMN was mixed with apoflavodoxin to give final concentrations of 0.5 μ M and 6 μ M respectively in 20 mM Tris-HCl buffer pH 7 containing NaCl (o) or Na_2SO_4 (Δ) or in sodium phosphate buffer pH 7 ($\bullet$). Second order rate constants were calculated from the observed pseudo-first order constants.

The rate constants for the binding of riboflavin (41.8×10^5 $M^{-1} s^{-1}$ in 100 mM sodium phosphate, pH 7) were higher than those for FMN binding, suggesting that the side chain of FMN sterically hinders the access to the binding site. Dissociation of riboflavin is also much faster, suggesting that the phosphate of FMN stabilizes the complex. The pseudo-first order constants for FMN and riboflavin binding showed a linear dependence on the concentration of apoprotein, and the line passed through zero, indicating that the reactions with both flavins occurred in a single step.

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