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LUMAZINES AS COENZYMES IN FLAVODOXIN: EVIDENCE FOR THE OCCURRENCE OF LUMAZINE SEMIQUINONES

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ABSTRACT

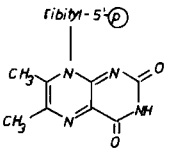
6,7-Dimethyl-N(8)-ribityllumazine-5'-phosphate 1, and 6,7-(2,3-dimethylbutano)-N (8)-ribityllumazine-5'-phosphate 2, bind tightly to apoflavodoxin from *Megasphera elsdenii*. The complexes are reduced by 0.5 equivalents of dithionite to form the corresponding semiquinones (λ_{max} ∿ 460 - 475 nm). An enzyme bound dihydrolumazine species cannot be obtained by direct reduction of the oxidized complexes; 7,8-dihydrolumazine is modified upon binding to apoflavodoxin (λ_{max} ∿ 400 nm) suggesting formation of a different tautomer, possibly the 5,8-dihydro form. The apoflavodoxin complexes of 1 and 2 show a catalytic activity, ∿ 30% and ∿ 90% that of the native enzyme in the photosynthetic NADP reduction of spinach chloroplasts. This allows the deduction that electron transfer in flavodoxins does not occur simply via the flavin C(8) position.

INTRODUCTION

Pteridine cofactors play a well established role in enzymatic hydroxylations of aromates[1] and methyl group transfer[2]. In addition to this, pteridine radicals have been suggested to play a role in biological processes[3], e.g. they have been implied to occur in photosynthesis[4]. The mechanism at molecular level of these enzymatic reactions, however, has not been elucidated in detail so far. The pteridine at its different oxidation levels, and pteridine radicals might be assumed to exist in different tautomeric structures[3,5,6,7]. In order to obtain more information about these possible intermediates we addressed ourselves to the questions of occurrence, stability and spectral characterization of pteridine related lumazines, at their 1 e^- and 2 e^- reduced states. In fact, ribityllumazine phosphate is structurally related to the flavocoenzyme FMN, and as such is bound by several apoflavoproteins[8]. The latter in turn are known to stabilize specific flavine radical forms[9] as well as the 1,5-dihydroform of reduced flavins[10]. Thus a similar stabilization of lumazine radicals and of specific dihydrolumazines bound to appropriate apoflavoenzymes might be expected which would then allow their spectral characterization. The similarity of the redox moiety of lumazines and flavins also suggests that they might be interchangeable as cofactors in some enzymatic reactions; specific steps in catalysis might thus become accessible to investigation, and allow deductions about the mechanisms of both classes of coenzymes.

RESULTS AND DISCUSSION

6,7-Dimethyl-N(8)-ribityllumazine-5'-monophosphate (1) and 6,7-(2,3-dimethylbutano)-N(8)-ribityllumazine-5'-monophosphate (4H-FMN) (2) bind tightly to apoflavodoxin from *Megasphera elsdenii*, a strictly FMN specific flavoprotein[10,11].



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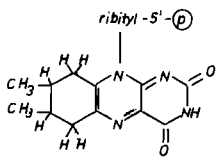

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Complex formation goes along with marked changes in the near UV portion of the lumazine spectrum, and with a quenching of the fluorescence emission to <10% of the original value. The spectral changes parallel those observed upon binding of the native cofactor FMN to the same apoenzyme[12]. From fluorimetric titrations, K_d values of $\sim 1.5 \times 10^{-8}$M in the pH range 5-7 were estimated for 1, while 2 binds with a K_d of $\sim 5 \times 10^{-9}$M at pH 7. These values compare with a K_d of $\sim 4 \times 10^{-10}$M for the binding of FMN to the same apoflavodoxin[12]. These observations indicate that the lumazine coenzyme analogs and FMN must be bound at the same place on the apoprotein and in a very similar fashion. Furthermore, comparison of the binding constants indicate that the aromatic flavin benzene moiety does not play a major role in the binding of FMN to apoflavodoxin.

The reduction of lumazines 1 or 2 with dithionite under anaerobic conditions is very fast, the 7,8-dihydro form being formed isosbestically with 2 reducing equivalents. When the same titration is attempted with the apoflavodoxin complex of 1, however, the decrease in absorbance in the 400 nm region, which indicates reduction, proceeds very slowly and might require up to 1 hr (depending on the amount of $S_2O_4^{2-}$ added) until equilibration is obtained. Up to the addition of ~ 0.5 molar equivalent of $S_2O_4^{2-}$ the reduction proceeds isosbestically and, in marked contrast to the reduction of free 1 or 2, goes along with the formation of a new species with λ_{max} at 470, 405 and 315 nm (Fig. 1). With the apoflavodoxin complex of 2 a similar species is formed with $S_2O_4^{2-}$. Admission of oxygen after the 1 e^- reduction completely and rapidly restores the oxidized lumazine spectra. Addition of further increments of $S_2O_4^{2-}$ causes only a very slow further reaction to occur which goes along with loss of the isosbestic points, but does not lead to complete disappearance of the long wave-length absorption. This indicates that the E_0' for the 2 e^- reduction of the complex must be considerably lower than that of free lumazine ($E_0' = -260$ mV)[13] and comparable to that of dithionite[14]. The 1 e^- reduced apoflavodoxin complex of 2 shows an unresolved EPR signal with a line width comparable

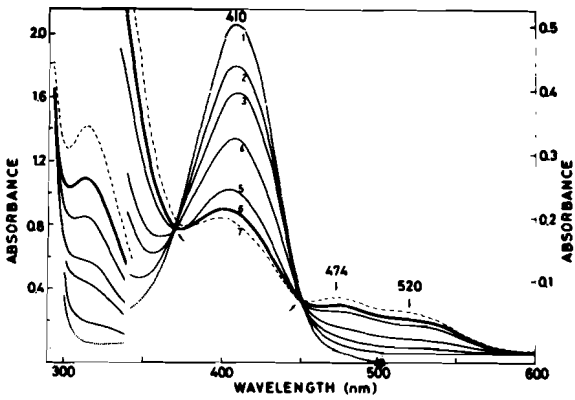


Fig. 1. Anaerobic reduction of the 6,7-dimethyl-N(8)-ribityllumazine phosphate (1)/ apoflavodoxin complex with dithionite. A solution of apoflavodoxin (1.5 X 10^{-4} M) in 0.01 M phosphate buffer pH 7.0 was incubated for 15 min at 0^{o} with an excess of lumazine phosphate (1) and the complex was separated on a short G-25 Sephadex column. Curve 1 shows the spectrum of the complex, 6.2 X 10^{-5} M in 0.1 M phosphate buffer pH 7.0 at 25^{o} and under anaerobic conditions. Curves 2-5 indicate the spectra obtained after addition of discrete amounts of a standardized solution of dithionite and after any time-dependent changes had gone to completion. Curves 6 and 7 represent the spectra obtained after addition of 0.50 and 0.575 equivalents respectively of reductant. The arrows indicate isosbestic points at 370 and 452 nm observed during the first phase of the reduction.

to that of native flavodoxin radicals. It should be pointed out that native flavodoxin radicals are formed analogously -- they have λ_{max} at 510 and 580 nm and are thermodynamically stabilized as compared to the free species[10].

From this we conclude that the long wavelength absorbing species indeed can be attributed to a (neutral ?) lumazine semiquinone. In good agreement with the radical spectra of Fig. 1 are the transient spectra obtained by Moorthy and Hayon in the microsecond range upon pulse radiolysis of pteridines[7].

Attempts to obtain complete reduction by photoirradiation in the presence of EDTA and catalytic amounts of deazaflavin[9] were unsuccessful as partial decomposition of the lumazine chromophore occurred. The complexes of the dihydrolumazines of 1 and 2 had thus to be obtained by a different approach, which relies on the expected kinetic lability of 7,8-dihydrolumzines towards tautomerism. Such experiments are outlined in Fig. 2. Lumazines 1 and 2 were first reduced with stoichiometric amounts of $S_2O_4^{2-}$, and then an excess of apoflavodoxin was added anaerobically. Marked spectral changes occurred immediately, and species with λ_{max} at $\sim$ 400 nm and a low intensity long wavelength absorption extending well over 500 nm were formed. Admission of air to these complexes lead to only very slow reappearance of the 410 nm absorbance, which is characteristic of oxidized lumazines. Similarly, aerobic

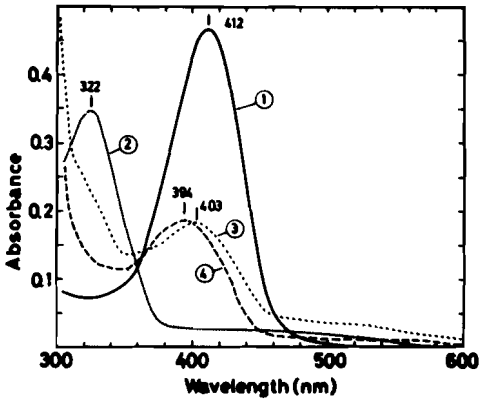
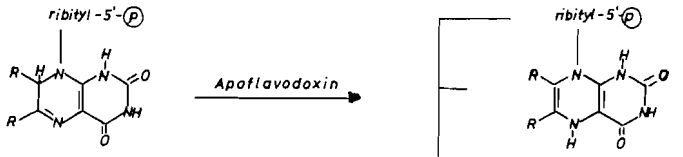


Fig. 2. Spectral changes accompanying binding of 7,8-dihydro-8-substituted "Lumazines" to apoflavodoxin from *P. elsdenii*. A solution of 4H-FMN, 4 X 10^{-5} M in 0.05 M phosphate buffer pH 6.0 was made anaerobic (Curve 1) and then reduced with 1.05 equivalent dithionite to yield the 7,8-dihydro-form (Curve 2). From a side arm of the cuvet a 1.2 molar excess of apoflavodoxin, 5 X 10^{-4} M in the same buffer was then added anaerobically. The resulting spectrum is shown by Curve 3. When the same set of experiments was carried out starting from 6,7-dimethyl-8-ribityl-lumazine phosphate the spectrum of Curve 4 was obtained. The curves are corrected for dilution. 4H-FMN was obtained by catalytic reduction of FMN as described for riboflavin[19].

denaturation of the complex by heat treatment caused only partial recovery of the absorption of 1 and 2. These spectral changes clearly indicate that the species bound by apoflavodoxin is not the 7,8-dihydro tautomer 3. That the 5,8-dihydro form is stabilized by apoflavodoxin is well conceivable, as in the native protein a hydrogen bond exists toward N(5), which stabilizes the blue radical[10]. This interaction could similarly induce a 7 → 5 tautomerism in the lumazine complexes. A dihydro pteridine with a long wave-length absorption, to which the 5,8-dihydro structure has been assigned, has been obtained by photodegradation of biopterin[16]. The lack of complete reoxidation of the complex might be attributed to the instability of intermediate reoxidation products either while bound to the protein, or upon release. However, alternative 5,6- or 6,7-dihydro tautomeric forms could exist as mesomeric structures, and could conceivably have absorptions in the 400 nm range, should also be considered as possible chromophores for the apoflavodoxin-bound species.

Nitrogen-(8)-substituted lumazines, and in particular 2, can be viewed as flavins lacking the benzene ring. This similarity might be expected within certain limits to result in an enzymatic activity when the flavocoenzyme is substituted by the lumazine analog. A case of particular interest, which would be accessible to experimental verification by such an approach is the proposal put forward recently, that in the



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electron transfer enzyme flavodoxin, exchange of redox equivalents occurs through the flavin position C(8)[17]. In fact, from X-ray crystallography data it appears that this part of the flavin coenzyme is readily accessible and exposed to the solvent, while the actual redox moiety in the pyrazine/pyrimidine part is buried into the protein[10]. In flavodoxin reconstituted with 1 or 2 a conjugation to such a position is missing. Furthermore with 2 the hydrogenated xylene moiety should constitute a steric barrier for an approach leading to contact between redox partner and redox center.

For activity tests the photosynthetic NADP-reduction system of spinach chloroplasts, in which ferrodoxin can be replaced by flavodoxin, was employed[18]. The apoflavodoxin complex of 1 was found to have 30% of the activity of the native enzyme under standardized conditions. With the complex of 2, when the lumazine and flavodoxin were mixed immediately before assaying, an activity ∿ 90% that of the FMN enzyme was observed. However, as will be detailed later (Ghisla, S. and Harzer, G., to be published) complexes of 2 are not stable and decay with a half time of ∿ 4 hr at 25°C; the modified complex shows no electron transfer activity.

CONCLUSIONS

The results outlined above confirm the expectation that an apoflavodoxin can recognize a different redox system as a coenzyme when specific structural elements are maintained such as a 5'-phosphorylated ribityl side chain and an intact pyrimidine moiety. Binding to the protein induces a markedly different chemical reactivity into the pteridine system. Thus lumazine semiquinones show a remarkable stability when bound to apoflavodoxin, and could first be characterized spectrally. Similarly, a dihydrolumazine tautomer is being stabilized which is clearly not the usually stable 7,8-dihydro form, and which is tentatively suggested to be the 5,8-dihydro isomer. The reduction experiments carried out with dithionite indicate that the redox potentials of lumazines bound to apoflavodoxin are modified in a manner very much similar to those of the native coenzyme[10]; i.e., the radical form is markedly stabilized, while the potential for the second reduction step must be drastically lowered. This potential might be in the range of that of native enzyme (-420 mV[10]) as indicated by the relatively high activity found with the reconstituted enzymes

as compared to native flavodoxin. With regard to the mechanism of electron transfer in flavodoxins, clearly the simple hypothesis that electron transfer between the redox partners requires involvement of the flavin C(8) position in catalysis[17] does not hold. Other mechanisms for electron transfer remain to be investigated. Possible alternatives include conformational changes occurring at the flavin side to allow in plane contact with the redox system or electron tunneling mechanisms.

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