First publ. in: Chemistry and Biology of Pteridines / ed.: John A. Blair. Berlin : de Gruyter, 1983, pp. 693-698

STUDIES WITH OLD YELLOW ENZYME, RECONSTITUTION WITH LUMAZINE ANALOGS AS COENZYME

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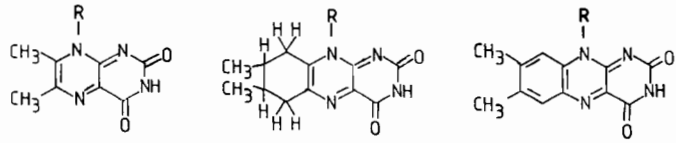
Abstract

8-Substituted lumazines can be regarded as "mutilated" flavins i.e. isoalloxazines lacking the benzene moiety. The 5'phosphorylated, 8-ribityl derivative binds to the apoenzyme of Old Yellow Enzyme with a K_d which is comparable to that of FMN (K_d 6×10^{-9}M, and 4×10^{-10}M for the analog, and FMN respectively). Binding induces profound spectral effects on the lumazine chromophore; this might be interpreted as reflecting a protein induced 7$\alpha \rightarrow$ 1 prototropy. The complex shows approx. 10% of the catalytic activity of native enzyme, in the oxidation of NADPH. Binding of aromatic molecules known to result in pronounced charge transfer spectra with native old yellow enzyme, have no spectral effect with the analog. These results indicate that the benzene moiety is important for the spectral effect, but not for catalysis.

Introduction

Derivativs of pteridine are the redox cofactors of an important class of enzymes which catalyze hydroxylations of aromatic compounds and methyl group transfer. They have evident chemical and structural analogies to the flavin cofactors: Both systems share a fused pyrimidine, and pyrazine moiety, the major differencies lying in the substitutions at the positions 2,6,7, and 8 of the pteridine. 10-Substituted lumazines (A,B) can thus be regarded as flavins (C) "mutilated" by the benzenoid subnucleus:



A B C

A further analogy between the two cofactor classes resides in the biosynthetical pathway, both cofactors are synthesised from GTP via, at least in the first steps, similar transformations. Both coenzymes catalyze the hydroxylation of aromatic compounds and activate molecular oxygen via formation of angular peroxides at the analogous flavin and pteridine positions 4a |1|. It is thus conceivable that mechanistic information which is available from one system, might help understand the other, and that the exchange of a pteridine cofactor in a flavin enzyme might be particularly fruitful. The natural cofactor of flavodoxin, FMN (C), has recently been replaced with a lumazine analog to yield a catalytically active enzyme |2|. Such studies have indeed yielded valuable mechanistic and structural information. Accordingly we have attempted the incorporation of flavin-like, pteridine derived coenzymes into Old Yellow Enzyme (OYE), an NADPH dehydrogenase, which belongs to a different flavoprotein class than flavodoxin, a typical 1e- electron carrier protein |3|.

Results and Discussion

4HFMN (6,7-(2,3-dimthylbutano)-N(8)-ribityllumazine-5'-monophosphate, Structure B, above) was synthetised as described earlier |2|, and was purified by reverse phase HPLC. The spectrum of free, 8-substituted lumazine is characterized by a single, strong absorption in the visible, and near UV, maximal at 410 nm (Fig. 1). Binding to the apoenzyme of OYE has profound effects; the visible maximum is shifted to 390 nm, and a new band of strong intensity appears at 320 nm. Such a pronounced effect is clearly unusual in its magnitude, for a simple protein interaction. On denaturation of the protein, most of the absorption of the cofactor is restored indicating that no irreversible chemical transformation has occurred (Fig. 1). A similar, however much slower process which was accompanied by loss of activity was observed earlier upon binding of 4HFMN to apoflavodoxin |2|. This prompted the suggestion, that there it reflects a protein induced $7\alpha \rightarrow 1$

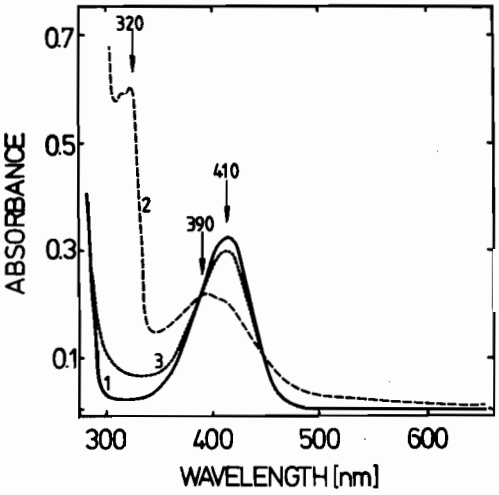
prototropic shift. Addition of phenolates, which form characteristic charge transfer complexes with native OYE |4|, do not cause major spectral perturbances with the artificial complex.

That the complex of 4HFMN and OYE is redox active is shown by Fig. 2. Anaerobic addition of 1 electron equivalent does not lead to formation of a radical absorbing in the 450-600 nm region, as was observed with 4HFMN-flavodoxin |2|. These results should be compared with those obtained with native flavodoxin, where a thermodynamically stable (blue) neutral radical is formed, and with native OYE which yields a labile (red) radical anion "5|. Thus the species of Fig. 2, curve 2, might have the structure of a lumazine radical anion. Further addition of redox equivalents leads to formation of a species with a maximum at 390 nm, which is significantly different from the oxidized form; readmission of oxygen restores essentially the spectrum of the starting material. The spectrum of the fully reduced species is closely similar to that, which was found to be stabilized upon binding to apoflavodoxin |2|, and is different from the product of dithionite reduction of free 4HFMN, i.e. from the 7,8-dihydro form the spectrum of which is shown on Fig. 3. When apo OYE is added anaerobically to this latter tautomer, no major spectral changes occur even after prolongued incubation suggesting that this species is the preferentially stabilized one also on the enzyme, or that the activation energy for tautomerization is very high. Denaturation of the protein under aerobic conditions restores most of the 400 nm absorption indicating that no major decay has occurred upon binding. These results indicate that the form obtained upon reduction of the enzyme bound 4HFMN is not the 7,8-dihydro tautomer, but, most probably, the 5,8-one, as was the case with flavodoxin |2|.

Fig. 4 shows that the 4HFMN complex is catalytically active in the transfer of redox equivalents from NADPH to oxygen, the only known activity of native OYE. The activity found is 10% that observed with native OYE and cannot arise from FMN or other flavin impurities. This implies that 4HFMN is bound probably in the same (1,,5-) paraquinoid form as normal FMN, and that the basic interactions with the protein backbone are the same as within the native enzyme. If the spectral changes discussed above reflect an $7\alpha \rightarrow 1$ prototropic shift, then the finding of catalytic activity would imply that also this "non paraquinoid form" is susceptible to reduction by reduced pyridine nucleotide, a question of relevance with respect to the mechanism of reduction of dihydrofolate by dihydrofolate reductase.

Fig. 1) Reconstitution of the holoenzyme of Old Yellow Enzyme with 4HFMN

Two ml of a $3x10^{-5}$ M solution of 4HFMN in 0.01 M phosphate buffer, pH 7.0 was incubated for 1.5 hrs at 4° with a 1.2 molar excess of apoenzyme. The sample was then applied to a G-25 column, and eluted with the same buffer. Practically all the 4HFMN was bound by the protein, as evidenced by the lack of free cofactor in the eluate. Curves (1) and (2) show the spectra of free and enzyme-bound 4HFMN, corrected for dilution, corresponding to a concentration of $2.8x10^{-5}$ M. The sample of curve (2) was then denatured with 5% (final conc.) trichloroacetic acid, and the protein precipitate was separated by centrifugation. Curve (3) shows the spectrum of the supernatant, after adjusting to pH 7, and correction for dilution.



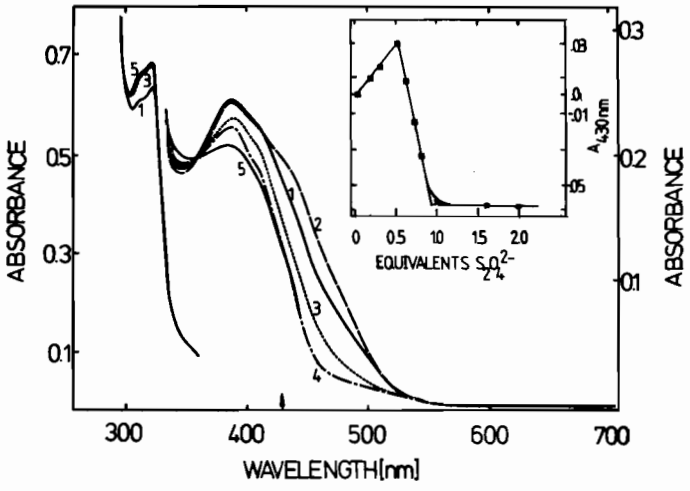


Fig. 2) Dithionite reduction of 4HFMN/Old Yellow enzyme

The artificial enzyme, $3.3x10^{-5}$M, in 0.01 M phosphate, pH 7.0 (curve 1) was titrated with aliquots of a standardized dithionite solution. Curves 2-5 show the spectra obtained after addition of 0.5, 0.7, 1.0, and 1.6 equivalents of dithionite. The inset shows the change in A_{430} during reduction.

Fig. 3) Formation of Holo Old Yellow Enzyme from reduced 4HFMN, and apoenzyme

A solution of 4HFMN, 2.5×10^{-5}M in phosphate buffer, pH 7.0 was made anaerobic (curve 1), and then reduced with 1.05 equivalents of dithionite to yield the spectrum of curve (2), i.e. that of the 7,8-dihydrolumazine chromophore. To this solution, a 1.3 molar excess of apoenzyme was added anaerobically to yield the spectrum represented by curve (3). This sample was then separated from excess cofactor, denatured and worked up as described in the legend of Fig. 1. Curve (4) shows the spectrum of the supernatant corrected for dilution.

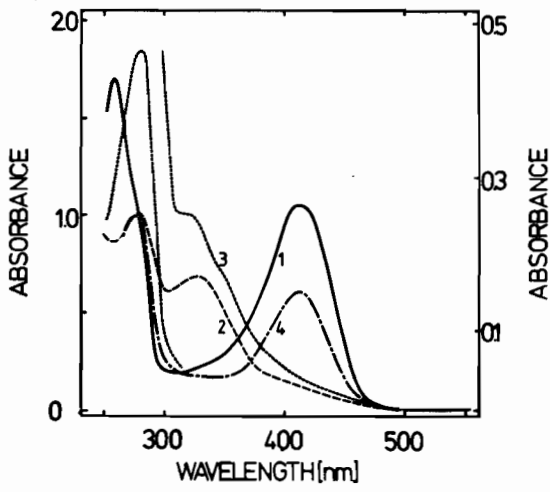
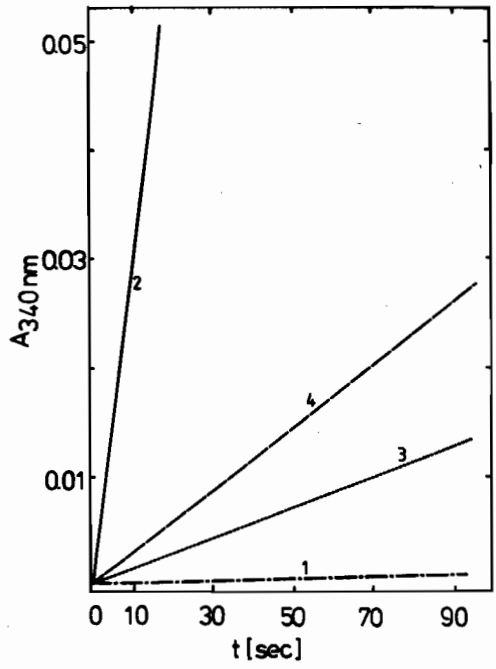


Fig.4) Demonstration of catalytic activity for the 4HFMN/Old Yellow enzyme complex.

The assay was carried out in a solution containing 5×10^{-5}M NADPH in 0.01 M phosphate pH 7.5, and at 20°. Curve 1 shows the changes of the optical density at 340 nm in the presence of 5.4×10^{-6}M apo Old yellow enzyme. Curve 3, and 4 show the results of the assay containing the same concentration of apoenzyme, which, however, had been preincubated for 4 min with 2.7×10^{-6} M, and 5.4×10^{-6} M (final concentrations) of 4HFMN. Curve 2 shows the disappearance of the NADPH absorption obtained with the same apoenzyme incubated with 1.4×10^{-6} M normal FMN. Note that the vertical scale represents negative absorbance changes.



Conclusions

The results discussed above, and analogous ones reported earlier |2| allow the following deductions:

a) The benzene moiety is not of primary importance for the binding of the coenzyme to apo OYE, and for catalytic activity. It however affects the formation of charge transfer complexes, and might thus play a role in the orientation ot the substrate(s) at the active site.

b) The activity of the artificial enzyme suggests that the lumazine system must basically be capable of undergoing the same type of enzymic reaction as the flavin, i.e. dehydrogenation, and activation of oxygen.

c) 8-Ribityllumazine precedes riboflavin in the biosynthetic path, and could be considered its "historical" ancestor. It also appears to possess comparable chemical and catalytical properties, thus the speculation might be justifiable, that it might have been a precursor of riboflavin derivatives as a cofactor. Evolutionary pressure could have favoured the use of the chemically more stable and versatile flavin system.

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