P-7 How and Why Are Some Riboflavin Coenzymes Covalently Attached to Proteins?

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I. INTRODUCTION

Most enzymes require cofactors for their activity. Some coenzymes of organic nature seem to be always present in a freely dissociable form (e.g., cobalamin, folic acid, niacin, phylloquinone, thiamine), others are known as covalently bound cofactors only, such as biotin and retinal. Several, however, have been observed both in free and covalently attached form (pantothenic acid and pyridoxin). In flavoproteins also, both modes of attachment are realized.

Fig. 1 Aminoacyl-flavin bonds observed in flavoproteins
Unlike the other covalent coenzyme/apoenzyme links where only one type of bond is realized for a given cofactor, flavin has been observed to couple to various aminoacyl groups of proteins and at different positions at the xylene ring (Fig. 1).

II. THE ABUNDANCE OF COVALENT FLAVOPROTEINS

The list of enzymes with covalent flavin attachment (Table 1) does not reveal an obvious relation between bond type and either the mechanism of the catalyzed reaction or the source of the enzyme. In terms of abundance in nature, the His(N3)-8α-FAD linkage appears to be prominent.

TABLE 1
The presently known covalent flavoproteins

| Enzyme                                      | Source                                      |
|---------------------------------------------|---------------------------------------------|
| **Histidyl(N3)-8α-FAD**                     |                                             |
| Succinate dehydrogenase                     | Mitochondria, Yeast, *B. subtilis*          |
| Fumarate reductase                          | *W. succinogenes, E. coli* (anaerobic)      |
| 6-Hydroxy-D-nicotine oxidase                | *Arthrobacter oxidans*                      |
| Choline oxidase                             | *Arthrobacter globiformis*                  |
| Dimethylglycine dehydrogenase               | Liver mitochondria                          |
| Sarcosine dehydrogenase                     | Liver mitochondria, *Pseudomonas*           |
| Sarcosine oxidase                           | *Corynebacterium* sp.U-96                   |
| D-Gluconolactone oxidase                    | *Penicillium cyaneofulvum*                  |
|                                             |                                             |
| **Histidyl(N1)-8α-FAD**                     |                                             |
| Thiamine dehydrogenase                      | Soil bacterium (ATCC 25589)                |
| Cyclopiazonate oxidocyclase                 | *Penicillium cyclopium*                     |
| Cholesterol oxidase                         | *Schizophyllum commune*                     |
| L-Galactonolactone oxidase                  | Yeast                                       |
| L-Gulonolactone oxidase                     | Liver microsomes                            |
|                                             |                                             |
| **Cysteinyl(S)-8α-FAD**                     |                                             |
| Monoamine oxidase                           | Liver mitochondria                          |
| Flavocytochrome c₅₅₂                         | *Chromatium*                                |
| Flavocytochrome c₅₅₃                         | *Chlorobium thiosulfatophilum*              |
|                                             |                                             |
| **Cysteinyl(S)-6-FMN**                      |                                             |
| Trimethylamine dehydrogenase                | Bacterium *sp.W₁A₁*                        |
| Dimethylamine dehydrogenase                 | *Hyphomicrobium X*                         |
|                                             |                                             |
| **Tyrosyl(O)-8α-FAD**                       |                                             |
| p-Cresol methylhydroxylase                  | *Pseudomonas putida*                        |

The simultaneous existence, often within the same organism, of covalently and non-covalently linked flavoproteins raises the question of the significance of covalency. Presently, the covalent flavoenzymes appear to be the minority. Can we expect to find many more? As far as microorganisms are concerned, the answer is very likely "yes". Inspection of Table 1 reveals that many covalent flavoproteins are engaged in the dissimilation of organic material. The potential of
many bacterial species to degrade oxidatively a wide variety of natural and artificial organic compounds is almost without limit. One might expect to find among the enzymes involved in the oxidative part of these catabolisms also flavoproteins bearing covalent flavin coenzymes. In cells of higher organisms, particularly in mammalian species, the number of covalent flavoproteins appears to be limited. Four covalent flavoproteins were distinguished in rat liver mitochondria and identified [1]: succinate dehydrogenase, monoamine oxidase, sarcosine dehydrogenase (sarcosine oxidase) and dimethylglycine dehydrogenase. Another covalent flavoprotein, L-gulonolactone oxidase, was discovered in liver microsomes [2].

The flavoprotein most widely distributed among aerobic organisms is succinate dehydrogenase (SDH). The corresponding enzyme of the anaerobic world is fumarate reductase. It does not only share the chemical reaction (though physiologically operating in the opposite direction) and the binding type of the apoenzyme with succinate dehydrogenase but also the genetic ancestry. The Sdh A subunit of SDH from beef heart mitochondria was the first example of an enzyme with the FAD attached covalently through a histidyl(N3)-8α-isooalloxazin linkage [3].

Several mutants of B. subtilis have been isolated that are unable to incorporate FAD into the SDH complex. They lack SDH activity, but membrane binding and assembly of the iron-sulfur centers are not affected [4]. The flavinylation of the flavoprotein subunit takes place before its assembly into the membrane-bound SDH.

III. THE MECHANISM OF COVALENT BOND FORMATION

The most intriguing problem following the discovery of various covalent flavoproteins is the mechanism of their synthesis. Covalent bond formation requires the expenditure of free enthalpy; in the case of biotinylation it is provided by ATP. Mechanisms involving ATP can also be formulated for covalent flavoprotein formation, but they would certainly require enzymatic catalysis. However, activation processes not involving ATP (or similar kinds of metabolic energy) or even enzymes can also be envisaged [5].

Non-enzymatic mechanisms need a particularly high conformational specificity of the binding region; furthermore, a base would have to be in a position to facilitate proton abstraction. The endergonic condensation reaction would be thermodynamically compensated by the reoxidation of the intermediately formed FADH2. Thus, this mechanism requires an oxidant capable of taking electrons from the reduced flavin. In contrast to the process involving a mono-oxygenase-type hydroxylation, however, it would not necessarily have to be oxygen.

The enzymology of covalent cofactor attachment is well known for the lipoyl- and biotinyl enzymes and for cytochrome c. Although the first instance of a covalent linkage in a flavoprotein has been discovered almost 30 years ago [3], the mechanism of the process has been elucidated only recently. Studies on 6-hydroxy-D-nicotine oxidase (6-HDNO) (for review see [6]) were the primary source of information. This flavoprotein is found in the aerobic D-nicotine-adapted soil bacterium Arthrobacter oxidans. The coenzyme, FAD, is bound to a His(N-3) of the polypeptide through its 8α-methyl group. The genetic information of this enzyme (Mγ = 49,077) is encoded on a 160 kb-plasmid, pAO1. Using pKK223-2 as vector the gene and its regulatory 5'-flanking regions were expressed in E. coli HB 101 and sequenced; Hisγ was identified as the site of FAD attachment. Usually, the flavinylation of the polypeptide occurs cotranslationally; however, conditions were found that allowed the accumulation of the apoenzyme and its purification [7]. In addition, a cell-free transcription/translation system of E. coli HB 101 was able to accomplish the formation of an enzymatically active 6-HDNO in the presence of FAD [8]. An efficient holoenzyme synthesis requires the presence of a low-mol.wt. compound, e.g. glycerol 3-P or phosphoenolpyruvate. These effector molecules do not participate in the reaction; they rather act as allosteric modulators of the flavinylation reaction that, most remarkably, does not require
an enzyme in addition to the apoenzyme (Fig. 2). Neither 8-Cl-FAD nor 5-deaza-FAD could substitute for FAD in holoenzyme synthesis. Site-directed mutagenesis revealed [10] that replacement of His$_{71}$ by serine, alanine or tyrosine allowed neither covalent FAD attachment nor enzymatic activity; cysteine in position 71 led to an enzymatically active holoenzyme with non-covalently bound coenzyme. This surprising finding suggests that covalent attachment \textit{per se} is not a necessary prerequisite of activity; rather the correct topology at the active center is important which can be accomplished in some enzymes best by covalent fixation of the cofactor.

The covalent flavinylation displays high specificity for both the protein and the cofactor. The "enzymatic" character of the reaction is further stressed by the requirement for an allosteric effector with certain structural features. It is of interest in this context that the cell-free flavinylation of the flavoprotein subunit of succinate dehydrogenase from \textit{E. coli} and of fumarate reductase also seem to need an allosteric effector; in this case it is a tri- or dicarboxylic acid, e.g. citrate, isocitrate, succinate or fumarate [9], again indicating functional specificity of the effector molecule. The flavinylation differs from any other enzymatic reaction in that the "enzyme" is also the substrate and the catalytic cycle operates only once!

IV. IS COVALENCY ADVANTAGEOUS?

1. Physico-chemical considerations. It is at present difficult to assign specific qualities to the covalent vs. the non-covalent flavoproteins and to correlate the binding mode with the functions of the enzymes. Neither the observed differences in the fluorescence properties nor those found in NMR studies of histidyl-flavins allow such assignments. Electron spin resonance measurements [11] showed the ionisation forms of the flavin semiquinones of all investigated covalent flavoproteins to be of the anionic type. Structurally, the presently known amino acid sequences adjacent to the binding group do not reveal a common denominator. Of course, it is possible that certain common features of the tertiary structure exist that characterize the FAD-binding domains of some covalent flavoproteins but are not evident from the short stretches of available amino acid sequences.
Similar considerations apply to the transition states in flavin catalysis. The firm attachment of the aromatic ring to the polypeptide backbone is likely to influence the mobility of the molecular structure of the coenzyme. This would be expected to reveal itself in several physical properties of the enzymes, most conspicuously in the redox potentials of the flavin/semiquinone and semiquinone/dihydroflavin couples. Measurements [12] of some histidyl(N)- and cysteiny1(S)-flavins as well as of the free forms (riboflavin and FAD) indicate that 8α-substituted flavins have a more positive (ca. 25 mV) redox potential than the free flavins. A significant difference between the various binding types does not exist. In view of the wide range of redox potentials of flavoproteins, it is difficult to attribute functional significance to the differences between covalently and non-covalently bound flavins, especially as the measurements were not taken on flavoproteins of comparable function but on model compounds.

2. Role of covalency in flavoprotein catalysis? Is covalent flavin binding to the apoenzyme correlated to the type of the catalyzed reaction? An unequivocal answer to that question cannot yet be given; in two instances only, succinate dehydrogenase and choline oxidase, is it obvious at the present time that the enzyme from many sources contains covalent FAD and apparently always of the same binding type; the similarity of the bond type even crosses the procaryote/eucaryote border. It may turn out that flavoproteins catalyzing a given reaction have the same kind of coenzyme attachment irrespective of the species. This, however, could reflect a genetic inheritance as well as a requirement of the reaction mechanism.

The 6-hydroxynicotine oxidases from Arthrobacter oxidans that catalyze the same type of reaction in the same organism but on the enantiomeric (D- and L-)substrates only [6], are genetically unrelated and use free and covalently bound FAD, respectively. Thus, the catalyzed reaction per se cannot be a decisive factor in the choice of coenzyme binding. It is questionable in that case, whether the stereospecificity of the reaction requires a specific type of cofactor binding. The apoenzyme of 6-hydroxy-D-nicotine oxidase is catalytically inactive and cannot regain substantial enzymatic activity by the sole addition of (free) FAD.

3. Covalency and turnover of flavoproteins. One aspect of covalency hardly discussed so far is the potential difference in metabolic stability between covalent and non-covalent flavoproteins. Cleavage of the aminoacyl-flavin bond is yet terra incognita. Indications have been obtained [6] that the FAD-free apoenzyme of 6-hydroxy-D-nicotine oxidase is more prone to proteolytic destruction than the holoenzyme. The faster destruction of the apoproteins may be part of an ordered intracellular turnover. Lacking information of systematic differences between comparable flavoproteins of the covalent and non-covalent type, conclusion as to an advantage of covalency cannot yet be deduced.

One should consider the possibility that covalency is advantageous under special circumstances only. Covalent bonding might be a means of cofactor economy under conditions of limiting nitrogen or riboflavin supply. Unfortunately, data on the half-life of covalent flavoproteins are not available and a correlation with non-covalent flavoproteins and with the generation time are not possible.

V. SUMMARY
Covalent flavinylation as elucidated by the formation of the histidyl(N3)-8α-flavin bond in 6-hydroxy-D-nicotine oxidase of A. oxidans proceeds by an non-enzymatic mechanism. Incubation of the apoenzyme, FAD and a three-carbon phosphate ester at neutral pH leads to the formation of an enzymatically fully active holoenzyme. The role of His71 in this process was illustrated by site-directed mutagenesis. Nevertheless, the question whether covalent attachment of the cofactor in a holoenzyme has prevailed throughout the evolutionary screening process because of biological significance or whether it is a chance event of neutral selective value [5] is still open.
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