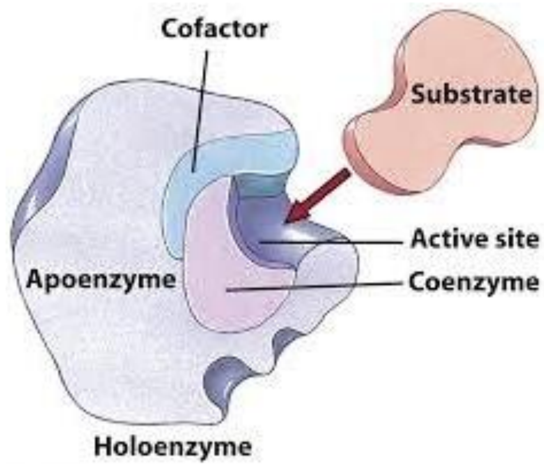
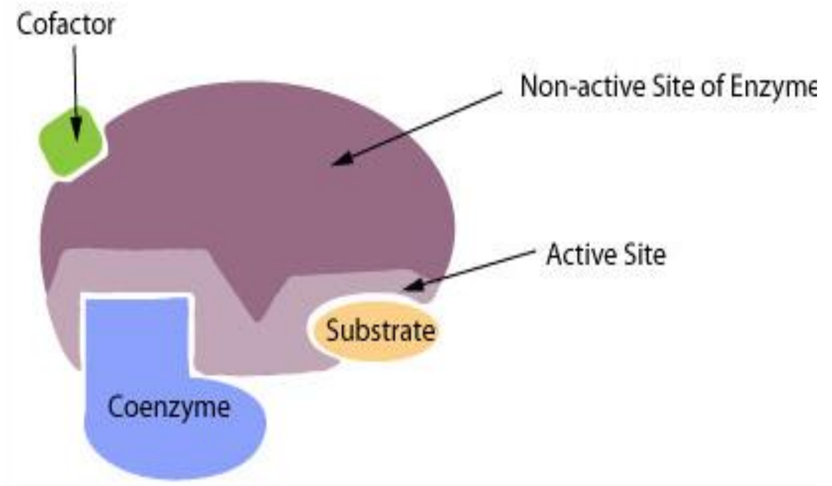
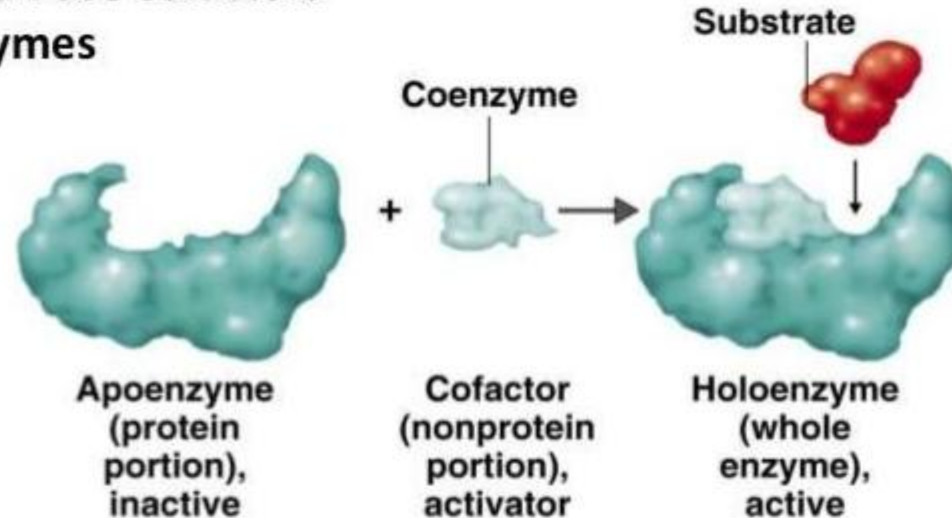


Coenzymes & Cofactor





- Some enzymes depend on their structure as protein for activity, while others also require one or more non-protein components for their activity ≡ cofactors.

- Cofactors may be metal ion or an organic molecule ≡ coenzyme. Some enzymes require both.

- The E-Cofactor complex is ≡ holoenzyme, and when the cofactor is removed, the remaining protein which is catalytically inactive ≡ apoenzyme.

- Although such cofactors may take p[art in the intermediate steps of the reaction catalyzed by the enzyme, they are not consumed during the process but are found in their original form at the end of catalysis. They may be regarded as the essential part of the catalytic mechanism.

- **Coenzymes were originally discovered as vitamins and growth factors in nutritional and medical studies. Most coenzymes are modified form of vitamins.**

- **Vitamins are trace organic substances that are vital to the function of all cells and required in the diet of certain species. Vitamins were originally discovered in nutritional studies, in which they were purified from food stuffs and shown to cure various disorders in animals maintained on deficient diet.**

- **Vitamins were discovered by their absence rather by their presence.**

Mode of Action

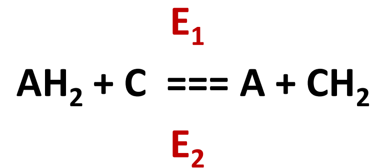
Majority of cofactors act in one of the following ways:

a) **As interenzymic carriers:**
b) **As intraenzymic carriers**
c) **By changing the shape of the enzyme molecule**
d) **By subunit aggregation**
e) **As stablizers**
f) **As tempelates**
g) **As primers**
h) **As intermediates**

Cofactors acting as carriers

a. **Redox carriers**

A very important group of cofactors consists of substances which are reduced by one substrate and oxidized by another.



$$AH_2 + C \underset{E_2}{\overset{E_1}{===}} A + CH_2$$

C = carrier

E_1 and E_2 are enzymes

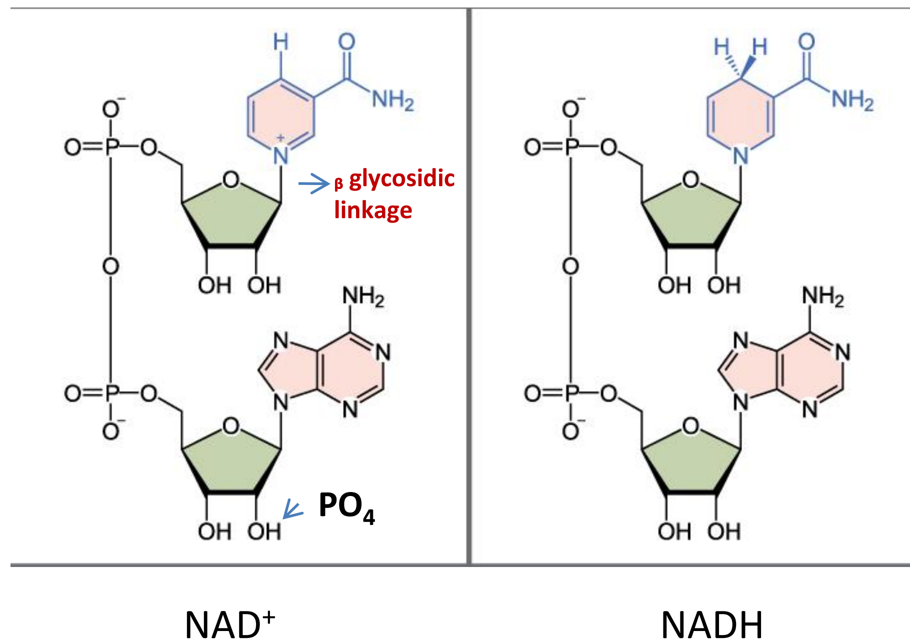
H atoms represent reducing equivalents

i. **NAD and NADP (DPN and TPN):**

-Two coenzymes are closely related and their existence has been known since many years.

The existence of thermostable coenzyme involved in fermentation [Coenzyme now identified as NAD] was shown by Harden and Young (1904) but it was not then isolated.

-It was isolated and purified by Von Euler et al and Warburg and Christian in 1936 independently.



▪Although NAD^+ is written as cation with one + charge at the nicotinamide gp., but in fact at pH 7.5, the two phosphates remain ionized giving one - charge on the molecule. While in $NADP^+$ the other PO_4 contributes to two more negative charges and the whole molecule bears three negative charges.

▪ NADP+ can be converted NAD+ by merely removal of the phospho group be alkaline phoaphatase.

Mode of Action

▪These coenzymes can be reversibly reduced by either by chemical reducing agents such as dithionite (somewhat slowly) or much more rapidly by dehydrogenases for which they are specific.

▪In the reduction two reducing equivalents per molecule are required.

- **It is nicotinamide ring which is involved in reduction, when the coenzymes are reduced, the uv absorption undergoes a change:**

-NAD and NADP show only a band at 260 nm due to purine and pyrimifdine ring.

-the reduced forms, NADH and NADPH, show an additional band at 340 nm. This band is due to quinoid bond structure of the reduced nicotinamide ring and is shown by no of simple derivatives of nicotinamide in the reduced state.

-Nucleotides which do not contain nicotinamide do not give this band.

-The transition between $NAD(P)^{+}$ = NAD(P)H is associated with the addition of one hydrogen atom and the removal of positive charge. Thus the oxidized is written as $NAD(P)^{+}$ and the reduced form as NAD(P)H.

-Although both forms are negatively charged and the transition is

$NAD^{-} = NADH^{--}$

$NADP^{--} = NADPH^{---}$

because of the –ve charges on the phosphate gps. However it is customary to ignore these charges and to write only the charges on the icotinamide part.

-When it is desired to write coenzyme without specifying oxidized or reduced form, NAD(P) is written.

MECHANISM

- **In the reduction of NAD(P) $^+$, one hydrogen atom and one electron are transferred to it, and these together form a hydride ion (H^-), the reaction together is referred to as hydride ion transfer. But it is by no means follows that these components are transferred as a single particle and one cannot deduce the overall mechanism from the overall reaction.**

- **NAD+ is reduced by a large no. of substrates in the presence of their specific dehydrogenases while NADP+ is reduced by fewer enzymes. Usually dehydrogenases specific for one coenzyme or the other, but some can use either by the two (though not necessarily equally well).**

- **Without enzymes the reduced forms of the cofactor are not oxidized at significant rates by O_2 , or by dyes such as methylene blue or by cytochromes; but they are oxidized by certain substances like phenazines, porphyrindienes and porphyrexides.**

- **Generally the dehydrogenases catalyze reversible reactions.**

- **The oxidation of NADPH by NAD^+ is catalyzed by $NADP^+$ transhydrogenase. Many of the reactions are rather specialized. A main function of these coenzymes is in the respiratory process . In mitochondria, the major reoxidation route for NADH is by way of ubiquinone and the respiratory chain.**

- **Another imp. Function is in anaerobic fermentation, where the reoxidation is due to other dehydrogenases acting in reverse.**

- **The stability of the oxidized and reduced forms vary with pH in opposite directions:**
    - **The reduced form is extremely unstable in acid but relatively stable in alkaline solns while**
    - **The oxidized form is fairly stable in acid but rather less stable in alkali**
    - **In neutral medium, the reduced form is less stable than the oxidized form.**

ANALOGUES

A no. of modifications of NAD and NADP have been prepared and studied with respect to enzymatic properties:

- ✓ **Changes in molecule, apart from the nicotinamide residue, like nicotinamide methiodide and nicotimade mononucleotide, are completely inactive with dehydrogenases.**

- ✓ **If adenine is replaced by nicotinamide in NAD, it becomes inactive.**

- ✓ **If NH2 of adenine is replaced by –OH gp. Giving nicotinamide hypoxanthine dinucleotide, the activity is reduced to the extent which varies with dehydrogenases.**

- ✓ **Modification of sugar residue also effects the biological activity.**

- ✓ **NAD containing deoxyribose in the adenylate residue show only low activity with dehydrogenases.**

- **Thus the adenylate half of the molecule is no less important than the NMN half for the coenzyme activity,**

- **In NADP, the position of third PO_4 gp is also important. In acid solution, this gp migrates spontaneously from 2’ to 3’ position, giving an analogue which is inactive with those dehydrogenases which are specific for NADPbut is active to some extent as NADP itself with most of those which are able to use both coenzymes.**

- **When the nicotinamide gp is attached by an α-link, the dinucleotide is inactive, as is also NAD with ring fully reduced.**

- **By replacing both bases with $NAD(P)^+$ nucleosides, a series of inactive analogues are formed.**

- **It has been seen that CO gp. Attached to the ring or some other unsaturated structure, is necessary for any coenzyme activity or even reduction by dithionite.**

- **Replacement of CONH2 by CSNH2 produces a dramatic increase of act. With some E but considerable fall in activity with others.**

- **Substitution or modification of NH_2 gp usually decreases but not abolish the activity, although replacement of -NH2 gp by $-CH(CH_3)_2$ produces an enormous increase with one E and complete abolition of activity with another.**

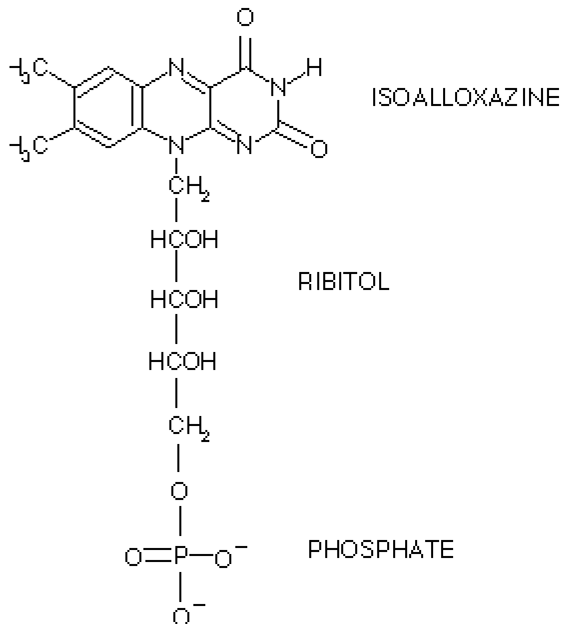
- **The specificity shown by the different enzymes differ greatly, and analogues have been used to differentiate enzymes from different spp. And even isoenzymes in the same spp.**

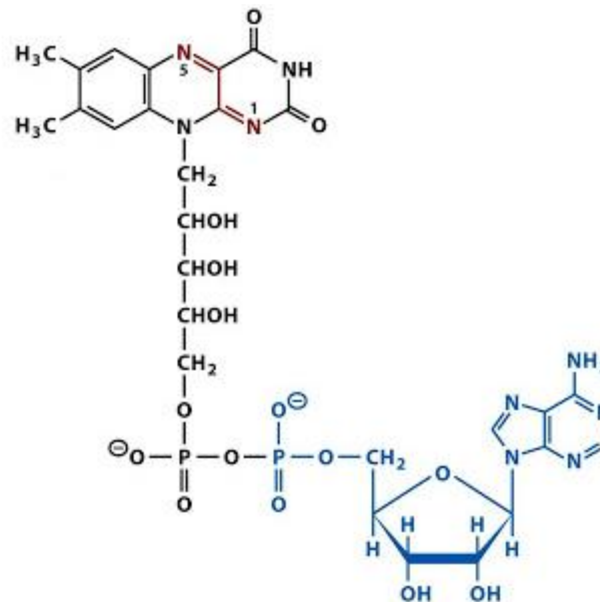
Other actions:

- **NAD and NADP act as cofactors where it is not obvious that oxidation processes are involved eg with lyases (4.11.35 and 4.2.1.45- 47)**
- **The isomerases (5.1.1.3 some egs) and**
- **The thansferases (2.12.10) which however dose not involve a reduction**
- **Some other nucleotides have the property of bringing about assembly of inactive subunits of enzymes into complete active enzymes molecules or acting as cofactor of type (D). NAD has shown this type of action too, apart from its ability to act as redox carrier eg.**
  - **Four inactive subunits of G6PDH of erythrocytes are aggregated in the form of tetramer active enzyme in the presence of NAD.**
  - **The same enzyme from *Neurospora,* NADP has been found to pevent the tetramer from dissociating into subunits.**

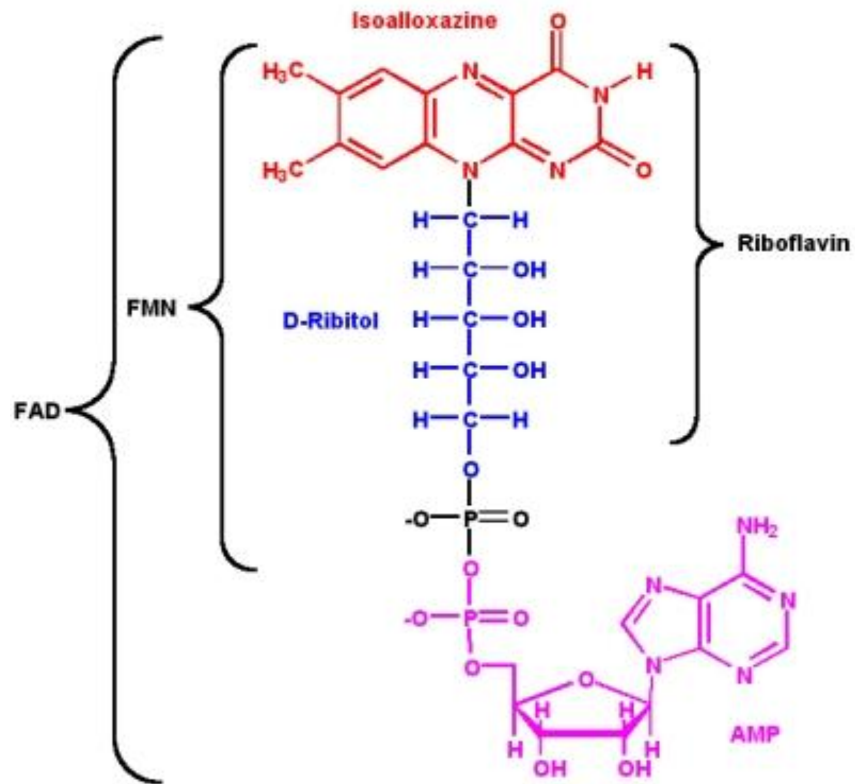
FLAVINS and FLAVOPROTEINS

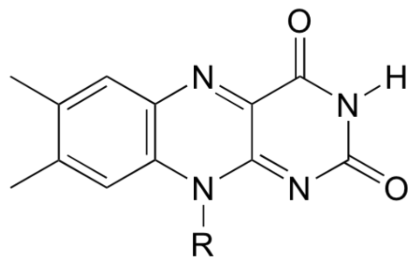
- The first flavoprotein enzyme, NADPH dehydrogenase [NADPH + acceptor = $NADP^+$+ $acceptor_{red}$ 1.6.99.1] was discovered by Warburg and Christian (1932) which they extracted from yeast and resolved into a yellow flavin gp. [Riboflavin-PO_4 or flavin mononucleotide] and colloid which was latter shown to be a protein.



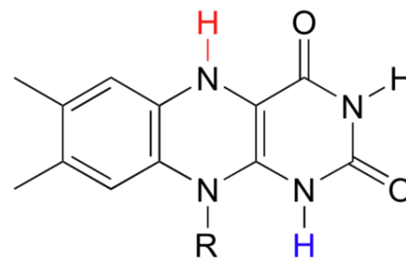


-The constitution of riboflavin was established by synthesis in 1935 by Kuhn et al and Karrer et al. Now no. flavin enzymes are known, a few of these have FMN as prosthetic gp. but the majority contain FAD.





FAD or FMN (oxidized flavin)



$FADH_2$ or $FMNH_2$ (reduced flavin)

- The oxidized forms are yellow fluorescent substances. These forms are readily reduced to the leuco forms by chemical reducers, and on reduction lose their yellow color and fluorescence, owing to the disappearance of the absorption band at 450 nm.

- The reduction involves addition of 2H atoms across the quinonoid structure in the isoalloxazine nucleus.

- Flavins bind to a no. of different specific proteins (apoenzymes) to give flavoprotein enzymes and generally one molecule of flavin is bound by each enzyme subunit. In majority of cases they are firmly and specifically bound to definite prosthetic gps although sometimes (eg in D-amino acid oxidase) the flavin may dissociate from the protein to some extent.

- The catalysis of redox reactions by flavoproteins is due to the alternate oxidation and reduction of their flavin gps.

- FAD differs from NAD, in that it completes its redox cycle while it is attached to one and the same enzyme protein, while NAD is reduced by one and oxidized by the other enzyme. FAD is therefore, an intraenzymic redox carrier.

- The properties of flavin are profoundly influenced by combination of the enzyme protein. In most cases the form of the absorption spectrum is not greatly changed on combination, although there may be slight modifications.

- The fluorescence properties of flavoproteins may be very different from those of the free flavins. Many flavoproteins do not floresce at all when oxidized and a very few show only a weak fluorescence (1-2% of that of the free flavins).

- The only one that has been found to approach the free flavins is dihydrolipoamide dehydrogenase (NAD^+). A small no. of flavoproteins have been found, unlike free flavins, to show some fluorescence in the reduced form only (upto 1% of FMN).

- The fluorecence has not been correlated wany of the property of the flavoproteins.

- The protein part of the flavoprotein enzyme determines both the mechanism and the specificity of the reaction catalyzed. Apoproteins have several important functions:
    - Provide substrate binding site which is responsible for the activation of the substrate and the high substrate specificity of the enzyme.
    - In many cases it carries metal ions (Fe, Mo, Cu) as part of its structure, and these may play a part in catalytic mechanisms, enabling the E to react with substances that do not reaction with free flavins (eg nitrate reductase plays through Mo).
    - The protein also has a stabilizing effect on certain half-reduced forms of the flavin, including semiquinones which play an imporatnt part in some of the reaction catalyzed.
    - Finally the protein part establishes a pattern of specificity for the flavin gp. towards the acceptors and therefore, determines functional class, to which the flavoprotein belongs

- **In the free state, reduced flavins show very little specificity and are readily oxidized by many acceptors.**

- **Combination with proteins, selectively prevents the reaction with particular acceptors; moreover, it appears that each protein imposes a different acceptor-specificity pattern on the flavin. Some of these effects are quite remarkable and is difficult to explain.**

- **Catalysis by flavoproteins depends on oxidation and reduction of their flavin gps, it is not always the fully oxidized and fully reduced forms that are solely involved. Half reduced forms of various kinds play an essential part.**

- **Three main types of mechanisms have been distinguished:**

i. depends on the oscillation between the fully oxidized and fully reduced forms of the flavins i.e.

F ==== FH_2 represented by glucose oxidase

here F is directly reduced to FH2 without formation of any intermediate (free radical semiquinone) and the acceptor oxidizes FH_2 directly to F.

Although the semiquinone form is not catalytically important, it is formed in large amount by reduction with dithionite, instead of glucose. It is also produced by NADH.

- In a variant of this mechanism, represented by cyt b5 reductase, F appears to be reduced to FH2 in one step by the substrate, but the oxidation of acceptor goes in two steps-

ii. Exemplified by dihydrolipoamide dehydrogenase (NAD^+): The oscillation is between the oxidized and the half reduced form, F → FH, and the FH_2 form is not involved in the calatytic cycle.

iii. Represented by cytochrome c reductase, F form is not involved in catalysis and the cyle involves the half and fully reduced forms, FH → FH_2. FH2 is oxidized to FH form by oxygen, ferricyanide, cyt c menadione but FH form is not oxidized further by these acceptors.

Thus the acceptor specificities of the fully reduced and half reduced forms of the flavin are different.

- In certain cases there is evidence that, in addition to flavins and metals, there are other goups in the apoenzyme that undergo oxidation and reduction during the catalysis.

- Particularly in the cases of dihydrolipoamide dehydrogenase (NAD^+) and glutathione reductase (NADPH), an S-S group in the protein is oxidized and reduced at the same time as the flavin and mechanisms have been proposed involving the formation of complexes of half reduced flavin and thiol group.