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Enzyme Reconstruction

Apoenzyme Reconstitution as a Chemical Tool for Structural Enzymology and Biotechnology

Ljiljana Fruk, Chi-Hsien Kuo, Eduardo Torres, and Christof M. Niemeyer**

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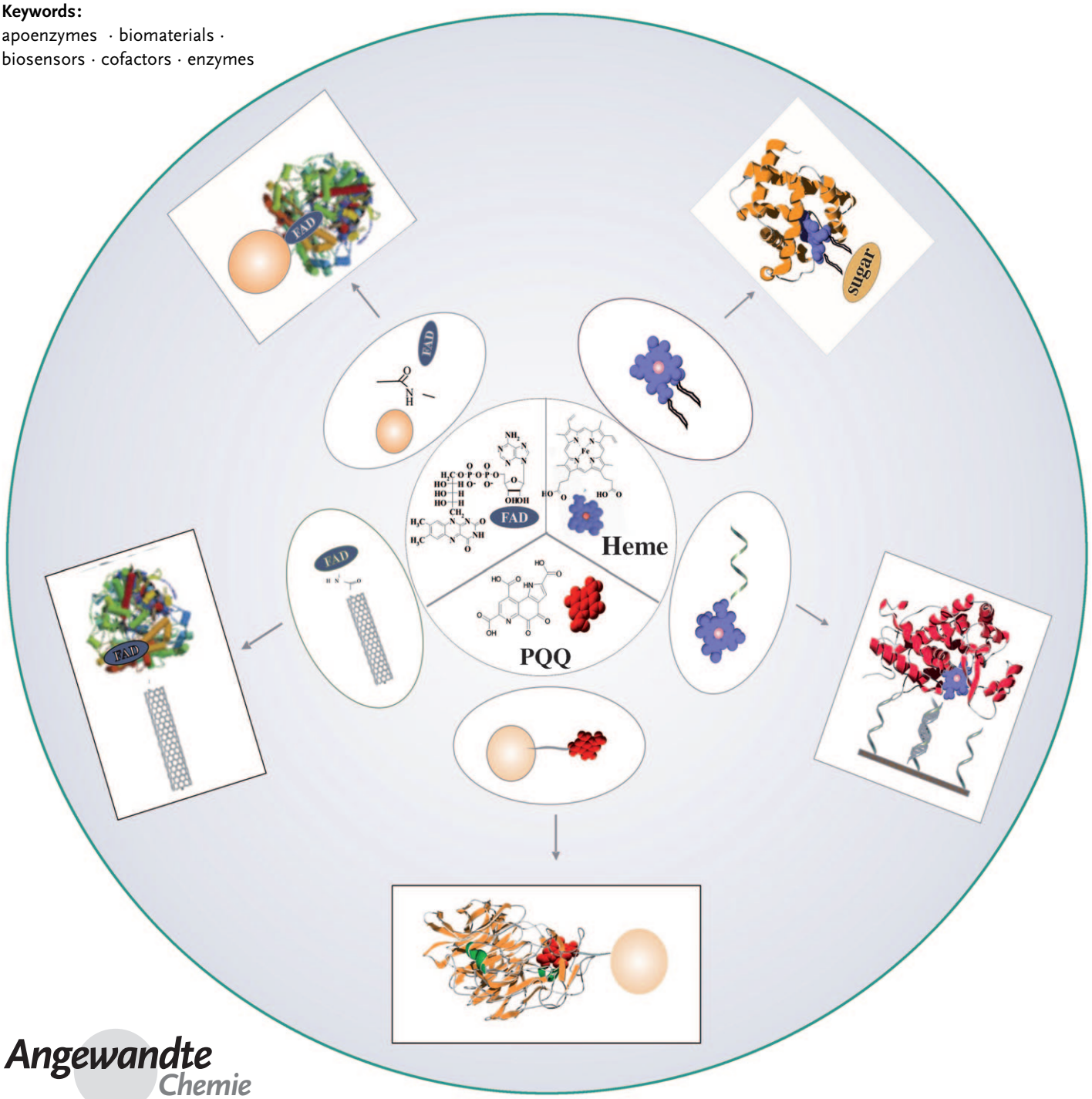


Many enzymes contain a nondiffusible organic cofactor, often termed a prosthetic group, which is located in the active site and essential for the catalytic activity of the enzyme. These cofactors can often be extracted from the protein to yield the respective apoenzyme, which can subsequently be reconstituted with an artificial analogue of the native cofactor. Nowadays a large variety of synthetic cofactors can be used for the reconstitution of apoenzymes and, thus, generate novel semisynthetic enzymes. This approach has been refined over the past decades to become a versatile tool of structural enzymology to elucidate structure–function relationships of enzymes. Moreover, the reconstitution of apoenzymes can also be used to generate enzymes possessing enhanced or even entirely new functionality. This Review gives an overview on historical developments and the current state-of-the-art on apoenzyme reconstitution.

From the Contents

1. Introduction

Many proteins contain a nondiffusible cofactor, often termed a prosthetic group, which is a nonpeptidic molecular moiety bound in the active site of the protein. Such a cofactor is required for the protein to conduct its function, such as the binding of substrates and other reaction partners as well as for the catalytic conversion. Prominent examples of prosthetic groups are porphyrin and flavin derivatives,[1] which are often involved in electron-transfer reactions. These derivatives can often be removed from the enzyme by chemical methods or biological manipulation, modified, and reinserted to obtain enzymes with different catalytic properties. This process is termed enzyme reconstitution. Figure 1a illustrates the reconstitution method, which includes the preparation of apoproteins. These are folded proteins which lack their respective prosthetic group, but into which naturally occurring or synthetic cofactors can be introduced. This approach represents a versatile method for investigating the reaction mechanisms of or introducing novel chemical functions into a given protein. In a broader sense, the term "reconstitution" also refers to the addition of metal cofactors (not only prosthetic group) into apoenyzmes, such as in the case of FeMo centers of nitrogenases[2–4] or NiFe centers of hydrogenases.[5] Although this approach is an exciting field in itself, in this Review we focus on the reconstitution of native and modified prosthetic groups with an organic framework.

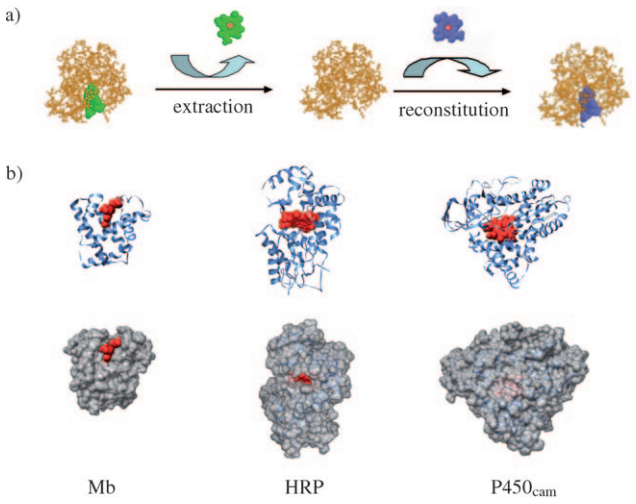


Figure 1. a) Schematic illustration of apoenzyme reconstitution. Extraction of the native cofactor (green) leads to the formation of an apoenzyme which can be subsequently reconstituted with an artificial cofactor (blue). b) Comparison of three different heme enzyme structures, with the heme cofactor indicated in red. The heme in Mb is positioned close to the surface of the protein, while in $P450_{cam}$ the reaction pocket is buried deeply in the tertiary structure of the protein. HRP is an example of a partially buried heme which can still be chemically removed. Note that these differences in the protein shell and the positioning of the cofactor account for the differences in the ease of cofactor extraction and reconstitution.

The reconstitution of an apoprotein with a non-natural cofactor moiety can be used for applications in (nano-) biotechnology, for example, the preparation of complex novel devices, such as nanoscaled scaffolds, addressable catalysts, or the fabrication of micro- and nanoarrays of semisynthetic proteins. Additionally, removal and re-insertion of a cofactor has been proven to be a powerful tool for structural enzymology, such as studying the role of specific atoms of the cofactor or of distinct amino acids in proximity to the prosthetic group in the active site of the protein. The latter is

[*] Dr. L. Fruk, C.-H. Kuo, Prof. Dr. C. M. Niemeyer
Universität Dortmund, Fachbereich Chemie
Biologisch-Chemische Mikrostrukturtechnik
Otto-Hahn Strasse 6, 44227 Dortmund (Germany)
Fax: (+49) 231-755-7082
E-mail: ljiljana.fruk@uni-dortmund.de
christof.niemeyer@tu-dortmund.de
Dr. L. Fruk
DFG Centre for Functional Nanostructures
Universitat Karlsruhe
Wolfgang Gaede Strasse 1, Karlsruhe (Germany)
Dr. E. Torres
Universidad Autónoma Metropolitana—
Cuajimalpa, Depto. Procesos y Tecnología
México, D. F. 01120 (Mexico)

particularly interesting in the case of heme enzymes, since the environment of the prosthetic group usually plays a crucial role in the enzymes activity.

In the past 30 years the reconstitution of, in particular, flavo and heme enzymes has been harnessed to enable electrical communication between enzymes and electrodes. This has allowed the elucidation of catalytic mechanisms as well as protein–protein and protein–ligand recognition processes, and the creation of novel biomaterials. From a synthetic chemistry and biocatalysis point of view, there is great interest in the design of novel, semisynthetic metalloenzymes which possess high selectivity and reactivity under mild conditions. Exciting progress has been made so that these semisynthetic metalloenzymes mimic their native counterparts. For example, heme enzymes with their metal-containing porphyrin cofactors are excellent scaffolds for the introduction of novel functions through reconstitution with artificial cofactors. This Review article presents an overview of the current state-of-the art of the reconstitution method as a tool to elucidate structure–activity relationships of enzymes and also to design novel biosensors and biomaterials.

2. Flavin Adenine Dinucleotide (FAD) Reconstitution

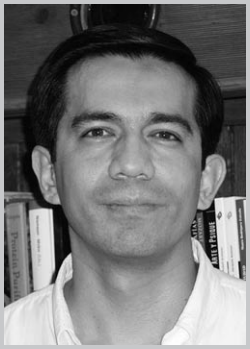
The cofactor flavin adenine dinucleotide (FAD, **1**) is a prosthetic group and cofactor found in numerous enzymes. It is involved in one and two electron transfer reactions in a number of biological processes, such as photosynthesis, the respiratory chain, and various metabolic pathways. The redox-active centers of FAD are located in the isoalloxazine ring, while the ribitol phosphate and adenosinephosphate moieties (Figure 2) mainly act as handles to stabilize the interactions between the cofactor and the amino acid residues of the protein. The specific interactions between the flavin and the protein, as well as the conformation of the FAD within the active site of the protein, define the catalytic activity of the enzyme. For example, the conformation of FAD can be elongated or bent (Figure 2a,b, respectively).[6] Only recently, FAD-containing proteins were divided and grouped into four different classes on the basis of a sequence–structure relationship according to specific relationships between their amino acid sequences and the folding conformation (Figure 3).[7]

2.1. FAD Removal

In the majority of flavoproteins, the cofactor is noncovalently but tightly bound to the protein scaffold, although recently a number of enzymes were discovered which contain FAD covalently bound through a histidine, cysteine, or tyrosine residue (Figure 4).[8] The nature of the binding mode determines the method of FAD removal. While several different methods have been developed to extract noncovalently bound FAD from holoproteins (fully functional and folded proteins), in the case of covalently bound FAD, the

Ljiljana Fruk studied chemistry at the University of Zagreb (Croatia) and completed her PhD in 2004 at the University of Strathclyde, Glasgow, where she worked with E. Smith and D. Graham on the design of SERRS probes for biospectroscopy. In 2004 she joined the group of C. M. Niemeyer, first as a Humbold Fellow and then as a Marie Curie Fellow, to work on the preparation of DNA–protein conjugates and their applications in biosensor design. Since 2008 she has been Research Group Leader at the Centre for Functional Nanostructures at the University of Karlsruhe, where she studies biofunctionalized nanoparticles.

Chi-Hsien Kuo received his BS from the National Taiwan University in 2000 and his MS from the National Tsing-Hua University (Taiwan) in 2004, where he worked on surface chemistry and investigated self-assembled monolayers on silica. In 2006 he joined the research group of C. M. Niemeyer as a PhD student after winning a DAAD scholarship. He is currently working on the reconstitution of novel heme enzymes.



Eduardo Torres graduated from the University of Puebla with a BS (1994), MS (1997), and PhD (2000) in biochemical sciences. From 2000 to 2006 he worked as a scientist at the Mexican Institute of Petroleum working with hemoproteins in the area of petroleum biotechnology, before joining the group of C. M. Niemeyer as a postdoctoral researcher in 2006. He is currently working at the Universidad Metropolitana-Cuajimalpa in Mexico City as a researcher in the Process and Technology Department.

Christof M. Niemeyer studied chemistry at the University of Marburg and received his PhD at the MPI für Kohlenforschung (Mülheim/Ruhr) with M. T. Reetz. After postdoctoral work at the Center for Advanced Biotechnology in Boston (USA) with C. R. Cantor, he habilitated from the University of Bremen in 2000. Since 2002 he has held the chair of Biological and Chemical Microstructuring in Dortmund. His research concerns the chemistry of bioconjugates and their applications in life sciences, catalysis, and molecular nanotechnology. He is the founder of Chimera Biotec, which is commercializing diagnostic applications of DNA–protein conjugates.

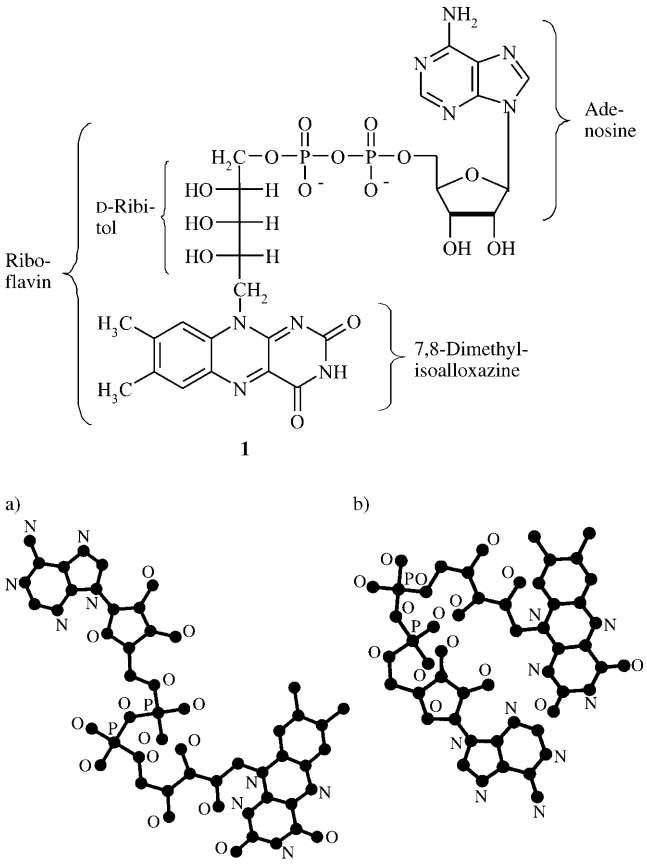


Figure 2. Structure of FAD and its a) elongated and b) bent butterfly conformation present in flavoenzymes.

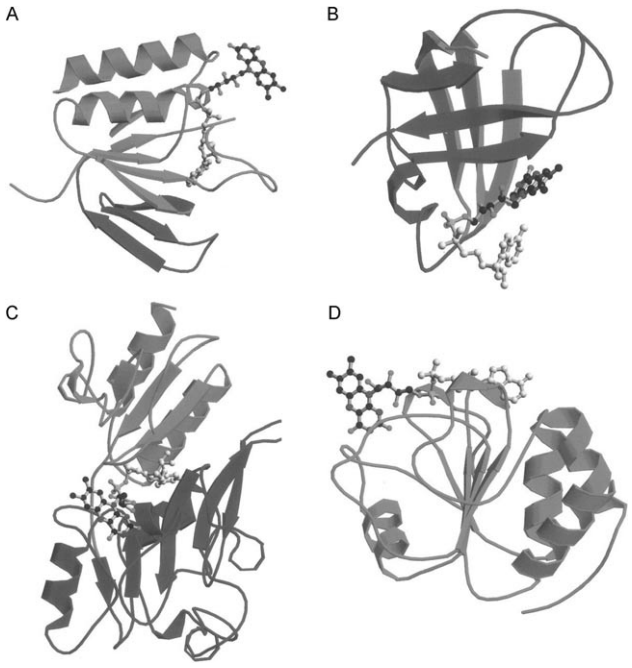


Figure 3. Four groups of FAD proteins based on the sequence–structure relationship. A) Glutathione reductase type; B) ferredoxin reductase type; C) *p*-cresol methylhydroxylase type; D) pyruvate oxidase type. Adapted from Ref. [7].

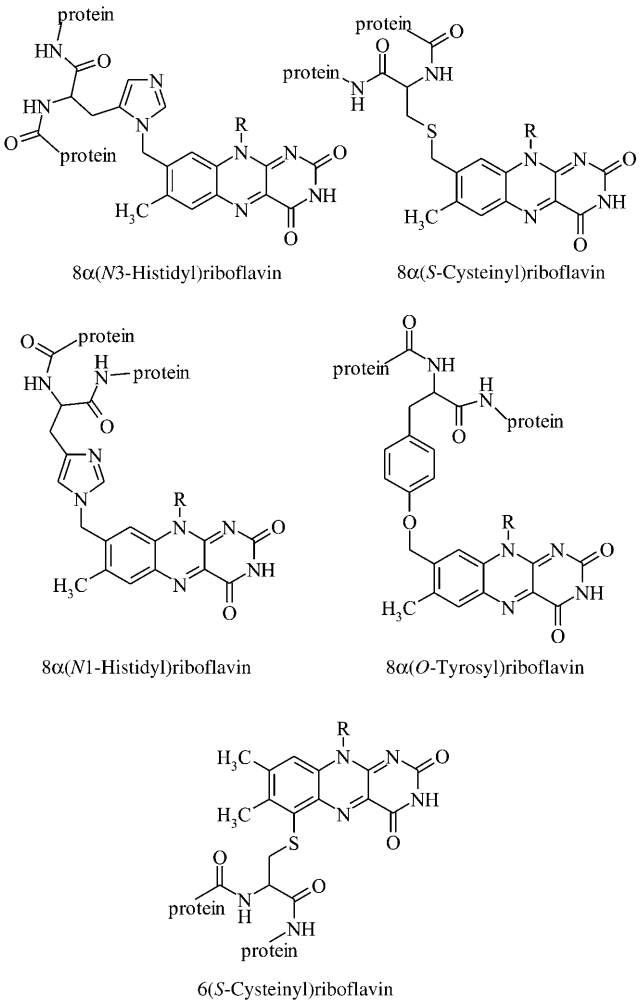


Figure 4. The structures of five known covalent linkages between FAD and protein.[9] R: ribityl-5′-diphosphoadenosine.

heterologous expression of the apoflavoenzyme is usually the only suitable method.[9]

The reports on the removal of the FAD cofactor from flavoproteins and their subsequent reconstitution go back as far as 1935, when Theorell used dialysis under acidic conditions to remove a yellow cofactor from the "Old Yellow Enzyme".[10] As flavoproteins greatly vary in their function and stability, a number of methods for the preparation of apoproteins has been developed, all of which have been adapted and optimized to the specific protein of interest. Early methods of flavin removal were based on the destabilization of the flavin–protein interaction at low pH values under high ionic strength and subsequent precipitation of the dissociated FAD.[11] Dialysis under non-native conditions has also been reported.[12] Recently, a number of chromatographic methods were developed to produce the apoenzymes by immobilization of the holoprotein, removal of the cofactor under slightly acidic conditions, and subsequent reconstitution.[13–16] The excellent review by Hefti et al. provides a detailed account on the advantages and disadvantages of various conventional and chromatographic methods, and it discusses briefly the importance of reconstitution-based

methods as a means to study structure–function relationships of flavoproteins.[17] Given that there are very informative review articles available that describe the historical developments of using artificial flavin cofactors[18] and surveying the role of FAD in the protein function and mechanism of flavin binding,[8,19] we will give here a short overview of the most recent advances in this field.

2.2. Flavin Reconstitution in Structural and Catalytic Studies

Reconstitution of apoflavoproteins with native,[20,21] artificial,[22–24] or isotopically enriched[25] flavins has been used for investigating the flavoprotein structure as well as mechanisms during redox catalysis. Recently, for example, the enzyme UDP-Galp mutase, which is involved in the synthesis of galactofuranose, was reconstituted with 1- and 5-deazaflavin derivatives (**2** and **7**, respectively, in Figure 5), thereby

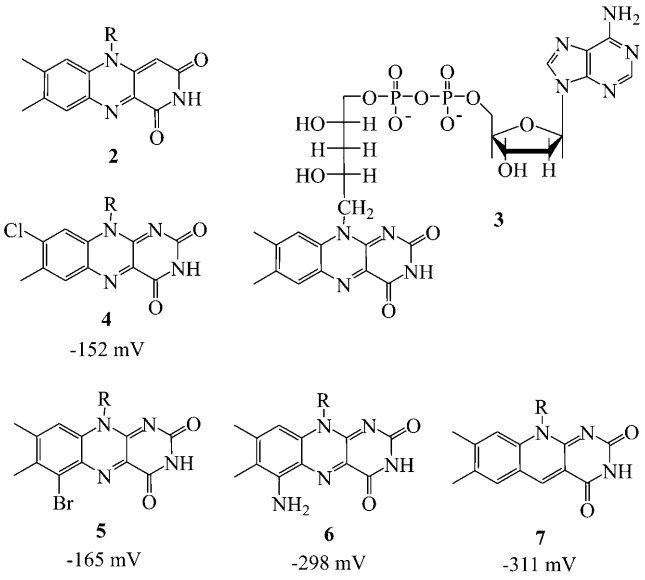


Figure 5. Artificial FAD cofactors used in reconstitution studies. FAD derivatives **4**–**7** were used for the reconstitution of pCMH and the investigation of its activity. R: ribityl-5′-diphosphoadenosine. The activity of pCMH increased with the redox potential (**4**→**7**).[37]

enabling the elucidation of its catalytic mechanism. It was found that the ring contraction reaction, namely the interconversion of UDP-D-galactopyranose (UDP-Galp) into UDP-galactofuranose (UDP-Galf), unexpectedly proceeds through the involvement of a number of radical species.[26] In another example, the role of the oxyanion hole in acyl-CoA dehydrogenase was probed by Raman spectroscopy by reconstitution of 2′-deoxy-FAD cofactor (**3**). This study led to the identification of possible mutation sites, which increase the enzymatic activity.[27] In another study, which attracted significant attention from the scientific community, the removal and in vitro reconstitution of superoxide reductase with native FAD was used to shed more light on the H_2O_2-dependent reduction mechanism of the enzyme.[28]

As mentioned above, a steadily growing number of flavoenzymes are being discovered which contain covalently bound flavin moieties. Their reconstitution with native and artificial cofactors has been used to investigate how the covalent linkage between the methyl group of the isoalloxazine ring and the protein residues is formed and whether it is important for the catalytic activity of the protein.[29–33] In a recent example, large amounts of soluble apoenzyme of monomeric sarcosine oxidase (MSOX) were produced by controlled expression in a riboflavin-dependent *Escherichia coli* strain. Its reconstitution with native flavin led to about 80% restoration of its native activity and to spectroscopic and catalytic properties indistinguishable from those of the native MSOX containing covalently bound flavin.[34] This study proved that the covalent bond between the cofactor and the protein scaffold can be formed subsequently to the insertion of the flavin into the apoenzyme, thereby confirming the results of other research groups on covalent flavinylation.[31,35] One of these investigations concerned the enzyme *p*-cresol methylhydroxylase (pCMH), which contains four prosthetic groups: two FAD and two heme domains. The investigations of FAD reconstitution by a recombinant apopCMH by Kim et al. led to them proposing in 1995 a mechanism for the formation of the covalent FAD bond. They found that the heme unit is necessary for both the formation of the covalent bond and the enzymatic function of FAD, because of its ability to store two electrons obtained from the reduced form of covalently bound FAD.[9] Later, Efimov et al. elaborated this mechanism and proposed that there are two phases in covalent flavinylation. Initially, rapid binding of FAD by the apoprotein (phase I) takes place which leads to an increase in the redox potential and subsequent covalent tethering of FAD (phase II) in its reduced form, which is capable of shuttling the electrons from FAD to two different heme domains of the enzyme.[36] In the case of the enzyme pCMH, it was shown that reduced FAD is significantly stabilized by covalent linkage with the protein scaffold. When 8-chloro- (**4**), 6-bromo- (**5**), 6-amino- (**6**), and 5-deaza-FAD (**7**; Figure 5) were used instead of the native FAD, it was observed that these derivatives can also be covalently linked to the protein and that this process mainly depends on their phase I redox potential.[37] It was also observed that the activity of pCMH increased with the potential of the bound flavin analogue (Figure 5). These findings might be used in the future to tune the redox properties and catalytic activity of flavoenzymes with covalently bound prosthetic groups.

2.3. Bioelectronics and Nanobiotechnology

The examples in Section 2.2 illustrate how cofactor reconstitution can be applied to investigate the structure and function of redox enzymes. This approach has also been widely used as a means to generate novel devices for emerging areas of sciences, namely bioelectronics and nanobiotechnology. Bioelectronics is a rapidly growing field which aims to integrate biomaterials in electronic devices by using complex biomolecules to fabricate transducers and read-out systems for the development of novel biosensors.[38–40] The

basis of such bioelectronic systems is the detection of electrons, which are transferred between electronic elements and the biomolecules during their signaling and catalytic processes. Thus, one key issue in bioelectronics is to ensure the electrical contact of biomolecules (very often redox proteins, such as flavo- or heme-containing enzymes) with electrodes to facilitate direct electron exchange. According to Marcus theory of electron transfer, the distance between the donor and the acceptor is a critical determinant of electron-transfer efficiency. In the case of redox enzymes, the protein shell separates the redox center from the electrode, thereby often prohibiting their direct electrical communication. Various methods, such as embedding enzymes in conductive polymer films[41] or the use of diffusional mediators such as quinones or ferrocene derivatives,[42] have been explored to facilitate direct contacting. A concept for the reconstitution of apoenzymes with modified cofactors to enable direct enzyme–electrode communication was developed by Willner and co-workers to provide new ways for the fabrication of electrochemical biosensors.[43] To enable electrical communication, the proper alignment and spacing of the redox centers at the electrode surfaces must be accomplished to gain control over the orientation of the enzyme and thus improve the performance of the biosensor. To this end, Willner and co-workers applied the FAD reconstitution of apoflavoenyzmes to ensure direct electrode contact and electrochemical triggering of their activity.[39,44] For example, they replaced the native FAD of glucose oxidase (GOx) with ferrocene-modified FAD (**8**; Figure 6) to facilitate precise attachment of the reconstituted GOx and its direct communication with the gold electrode. This approach preserved about 60% of the original activity of the native enyzme.[43]

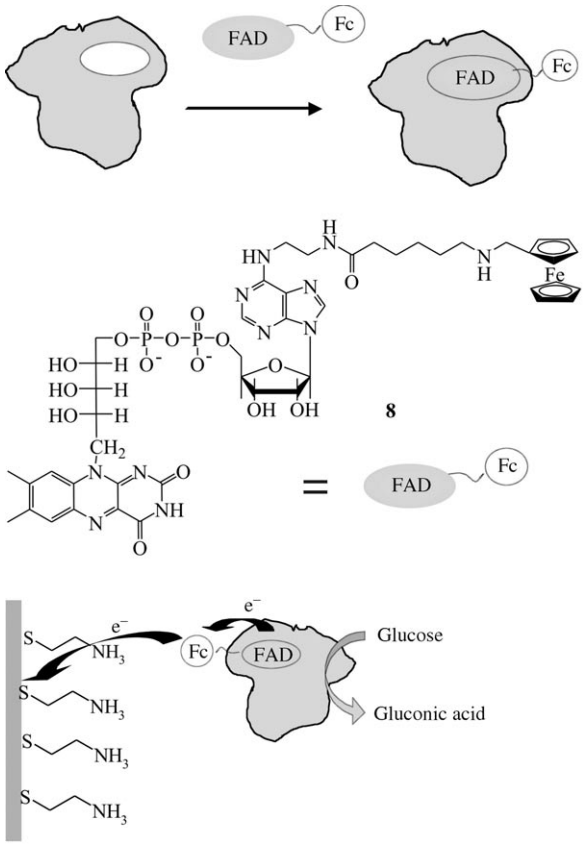


Figure 6. Reconstitution of apoGOx with ferrocene-modified FAD. The enzyme was subsequently adsorbed on gold electrodes. Adapted from Ref. [39]. The gray bar represents the gold electrode.

Subsequent work on GOx reconstitution elaborated the concept of Willner and co-workers, and has been surveyed in a number of review articles.[45–47] We will thus here focus on representative examples to briefly illustrate the state-of-the-art. The concept was elaborated further to improve the electrical communication by using various electrode modifications with relay linkers incorporating pyrroloquinoline quinone (PQQ),[48] phenylboronic acid derivatives,[49] rotaxane-type molecules,[50] functionilized polymers,[51] or modified FAD derivatives containing additional functional groups (such as amine **9**, Figure 7) to enable further functionalization.[40,52,53]

With the advent of nanobiotechnology, a field of research concerning the biofunctionalization of nanometer-sized systems and the use of nanosystems to elucidate biological systems,[45,54–56] the reconstitution of apoenzymes was used to generate novel nanosystems. In a demonstration of this approach, FAD reconstitution was utilized to bridge gold nanoparticles acting as electrical connectors between electrodes and the redox center of a biocatalyst (Figure 7a).[45,57] The incorporation of gold nanoparticles into the electrochemical system led to an up to sevenfold increase in the electron-transfer rate as compared to the native GOx connected to the electrode in the absence of the gold nanoparticles. The gold nanoparticles act as "plugged-in wires", thus enabling the direct electrical contact between the redox center of the enzyme and the electrode, and they might also function as nanocurrent collectors.[57,58] In a similar approach, the Au nanoparticles were exchanged with carbon nanotubes that were functionalized with FAD (Figure 7b). An in situ apoGOx reconstitution led to an increase in the coverage of the gold electrodes and excellent electrode–enzyme contact.[59,60] A sixfold increase in the rate of glucose oxidation was obtained, as compared to the control reaction with GOx only. A dependence of the amperometric response on the length of the connectors was observed when the oxidation of glucose was monitored electrochemically in the presence of nanotubes of different length, and indicated that shorter nanotubes (25 nm) facilitate faster electron transfer than longer ones (150 nm).[59]

In addition to GOx, other FAD enzymes such as glucose dehydrogenase (GDH),[51] D-amino acid oxidase,[43] and cholesterol oxidase (CHO)[61] have also been used successfully for reconstitution by Willner and co-workers to ensure electrical contact and allow activity measurements. These studies showed that the reconstitution of apoenzymes are a versatile and efficient way of electrically wiring redox enzymes to enable the development of amperometric biosensors and biofuel cells.[45–47,62] It can be envisaged that a detailed understanding of flavoenzyme function will facilitate a specific tuning of enzymatic activity and result in its application to the easier design of even more sophisticated devices for bioelectronics and novel hybrid materials. Some

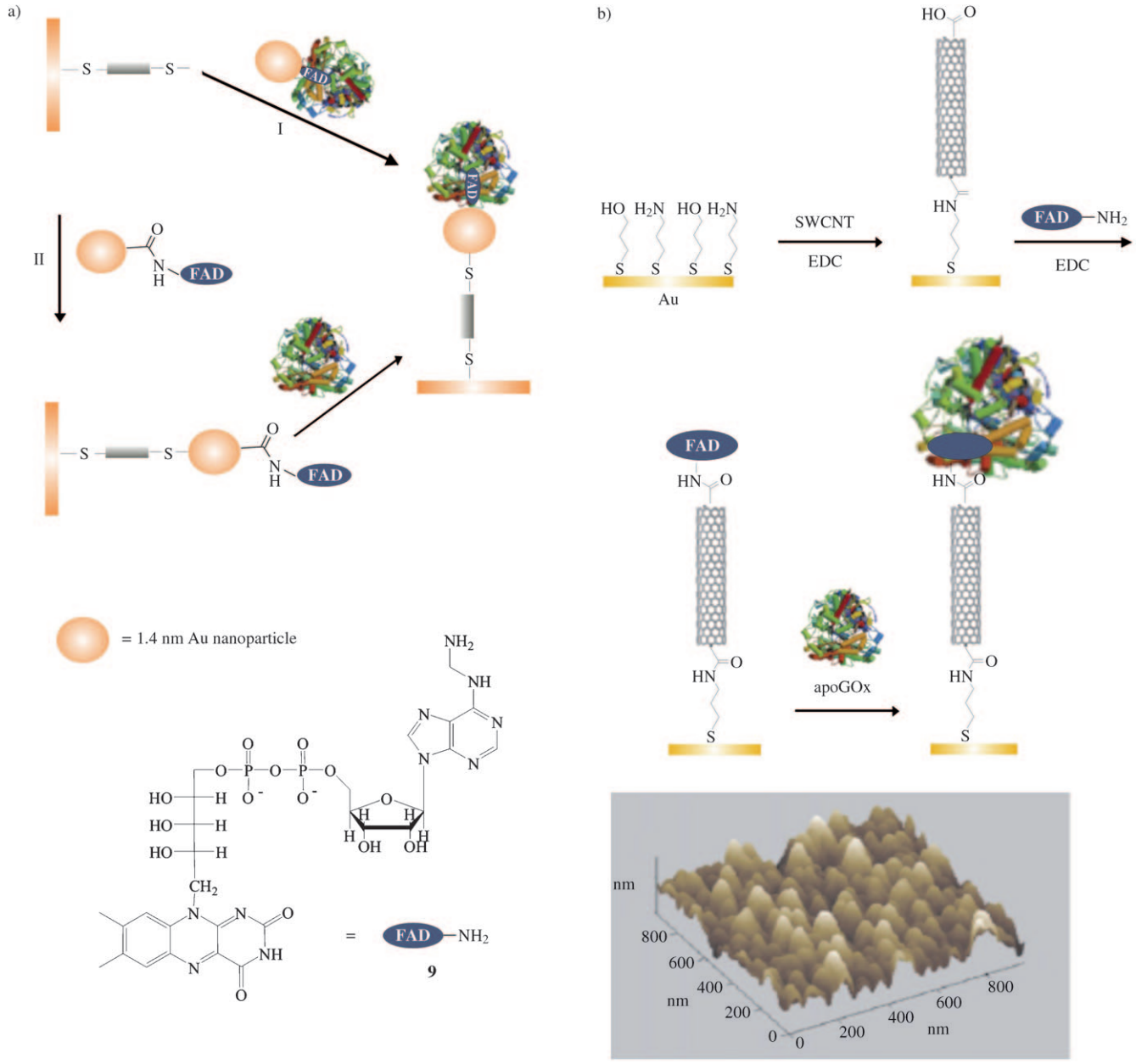


Figure 7. a) Preparation of GOx-modified electrodes using Au nanoparticles as electrochemical relays. I) Reconstitution of apoGOx with the FAD-modified nanoparticles and subsequent binding to the electrode. II) Binding of the FAD-modified Au nanoparticle and subsequent in situ reconstitution. Adapted and reprinted with permission from Ref. [57]. Copyright 2003 Science/AAAS. b) Reconstitution of apoGOx on gold electrodes containing single-walled carbon nanotubes (SWCNT) modified with FAD using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). The AFM image of the electrode coverage is shown at the bottom. Adapted from Ref. [59].

examples in this direction based on heme-containing enzymes are described in Scheme 3.

3. Heme Reconstitution

A large body of knowledge on the reconstitution of apoenzymes has been generated from heme enzymes. Thus, we will initially describe approaches to elucidate the mechanistic details of the catalytic activity of hemoproteins which will be followed by a discussion on concepts and examples of the design of semisynthetic heme enzymes. These studies will show that novel properties can result that are distinctly different from those of the native enzymes.

The three general biological functions of hemoproteins, for example, of the family of cytochrome P450 monooxygenases[63] and peroxidases,[64] concern the transport of electrons (for example, cytochrome b5), the transport of oxygen (for example, hemoglobin), and the catalysis of various types of metabolic reactions. Despite their different functions, all of these proteins possess an iron protoporphyrin IX moiety (heme, **10** in Figure 8) as the prosthetic group in their active

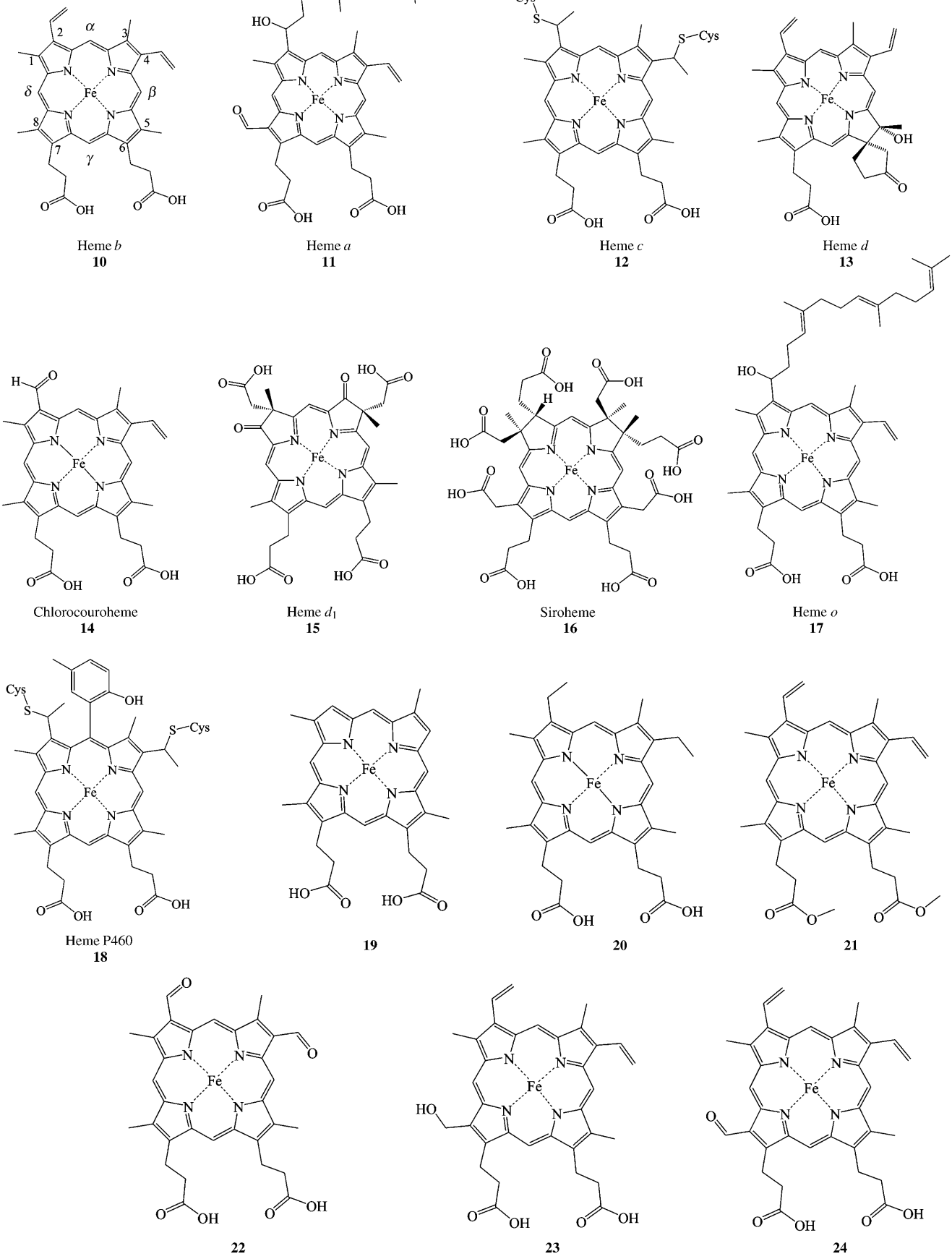


Figure 8. Structures of naturally occurring (**10**–**18**) and some synthetic heme cofactors (**19**–**24**) used in reconstitution studies.

site. The activity and efficiency of the enzyme often depend on the way the heme is positioned within the protein shell as well as on the interaction of the heme with the substrate. Understanding the mechanisms by which the intrinsic reactivity of the proteins is controlled is of both theoretical and practical interest. The catalytic properties of heme-dependent enzymes are governed by several factors, such as the structure and conformation of the prosthetic group, the ligands coordinated to the iron atom, the amino acid residues of the protein in the vicinity of the cofactor, as well as the general topological and physicochemical properties of the active site. Therefore, the exchange and/or replacement of the cofactor can provide valuable insights into many different aspects of the enzymatic activity and, as mentioned above, it can be used to introduce novel chemical functionalities into a given protein scaffold, thereby opening up new applications.

3.1. Removal of the Heme Cofactor

One of the first model enzymes used to study heme association and dissociation was myoglobin.[65–67] This small (17600 kDa) oxygen-storage protein (Figure 1b) is readily obtained from sperm (whale) or heart (horse, bovine). It is easy to purify and its folded tertiary structure is sufficiently stable to withstand removal and reconstitution of the heme group (**10**). The methodology for the removal and re-insertion of heme groups initially developed for myoglobin[68] has been used in numerous studies on other enzymes, and has led to a plethora of previously inaccessible data regarding their stability, structure, and function. In Section 3.2 some established protocols are presented as well as recent advances achieved by heme reconstitution.

Noncovalently bound heme can be extracted from the partially denatured[69] enzyme by following Teale's protocol.[70] Since the first publication, this method has been used for the extraction of heme from numerous enzymes, including myoglobin (Mb), horseradish peroxidase (HRP), haemoglobin (Hb), as well as various heme proteins from the cytochrome family[71,72] such as cytochrome *c* peroxidase (CCP)[73] and P450 enzymes (Figure 1b.[74,75] Teale's method is based on the acidification of a solution of the protein to induce denaturation, followed by extraction of the heme cofactor with organic solvents, such as 2-butanone (methyl ethyl ketone). The method is quick and applicable to even small volumes of enzyme solutions. Although acidic conditions are needed to partially denature the protein, many apoproteins produced by this method are sufficiently stable to be reconstituted with heme derivatives. However, the yield of the reconstitution varies greatly and depends on the structure of the protein under investigation, as well as on the position of the heme pocket within the three-dimensional structure of the protein.

As an example, apocytochrome *c* peroxidase (apoCCP) is stable enough to be crystallized;[73] however, interestingly, it is not possible to reconstitute apoCCP when the crystals are formed. This phenomenon is a consequence of the rigid conformation in the crystalline state and the inability of the heme to reach its binding site because of the large size of the heme molecule in relation to the size of the pores in the crystal lattice.[73] On the other hand, many proteins do not withstand the removal of the heme cofactor. For example, cytochrome $P450_{scc}$ could not be used for the reconstitution because the removal of the heme led to precipitation of the apoprotein, which could only be dissolved in 6 M guanidine hydrochloride or 10% NaOH upon heating. This treatment results in a solution of an enzyme which can not be refolded to produce functional holoenzyme.[76]

Milder methods of apoenzyme preparation have been developed to enable the removal of heme from enzymes which are too sensitive for the acid/ketone treatment. One of them is based on the incubation of the protein of interest with a solution of apomyoglobin (apoMb) or the protein hemopexin from human serum. In some cases this results in heme transfer from the protein of interest to the apoMb acceptor.[77,78] Since this method depends on the relative affinities of the two competing apoproteins, it has also been used to study the thermodynamics and kinetics of removal and reconstitution. In one such study, a mutant of myoglobin (H64Y/V68F apoMb) with a high affinity for heme was designed. This was used to quantify heme dissociation constants of a range of wild-type Mbs from different sources, as well as of Mb mutants in which distinct amino acids in the vicinity of the heme binding pocket were exchanged.[79,80] It was observed that one of the key factors leading to the stabilization of heme binding in Mb is the hydrophobic interactions between apolar residues in the heme pocket and the prophyrin ring. Moreover, distinct interactions between His93 and the Fe^{3+} ion, which had previously been pointed out by Rose and Olsen,[81] as well as hydrogen bonding between distal residues and coordinated water[82] appear to be crucial for binding of the cofactor. The dissociation of heme from the enzyme and the subsequent transfer of the heme to the high-affinity H64Y/V68F mutant apoenzyme was monitored by UV/Vis spectroscopy.[80] The same method has also been used to investigate the influence of distinct amino acids, in particular, histidine residues in the vicinity of the heme binding pocket, on both heme reconstitution and substrate binding.[79] Interestingly, in contrast to previous models, it was observed that the residues around the heme propionate groups have little impact on ligand binding and autooxidation.[79]

In contrast to various globins and cytochrome proteins, where the heme moiety is only partially embedded inside the polypeptide framework, in the P450 enzyme family the heme group is almost completely buried inside the tertiary structure of the protein (Figure 1b). It is thus rather difficult to reconstitute cytochrome P450s because the removal of the cofactor requires almost complete denaturation of the protein. Therefore, a careful adjustment of the extraction conditions is needed for the preparation of P450 apoproteins, and one of the successful approaches includes treatment with acidic buffers, as described by Correia and Meyer.[83] This method does not involve the extraction of the cofactor, but instead decomposes the heme group in situ by treatment with concentrated hydrogen peroxide or detergents in the presence of 1% β-mercaptoethanol and leads to yields of up to 90% of the reconstitutable apoenzyme.[83,84] While exploring

the removal of heme from P450 enzymes, Sadano and Omura observed that the half-life of the polypeptide chain of cytochrome P450s in vivo is significantly greater than that of the heme moiety.[85] This observation shed light on the turnover of the cofactor within the cell, since it suggested that cytochrome P450 enzymes may exchange their prosthetic groups several times during their lifetime.

The harsh conditions required for the preparation of P450 apoenzymes means that it is very difficult to reconstitute functional enzymes, and only a few reports have so far been published. As an example, Uvarov et al. used hydrogen peroxide to produce microsomal apocytochrome $P450_{LM2}$, and 50% of the apoenzyme was then successfully reconstituted to yield active enzyme.[76] The same method was applied to bacterial $P450_{scc}$, which was reconstituted with a heme derivative containing esterified propionic groups to study the effects of these groups on enzymatic stability and activity.[86]

In addition to the development of methods for heme removal, the mechanism of the re-insertion of this prosthetic group has attracted significant attention. Vasudevan and McDonald described four distinct phases in the reconstitution of apohemoglobin with heme, and they determined rate constants for each phase by using spectrophotometric heme titration.[87] The phases include heme insertion (phase I), local rearrangement of the protein structure (phase II), global conformational response (phase III), and the rate-determining phase, the irreversible formation of a histidine–iron bond (phase IV). In earlier studies, Rose and Olson were able to determine the equilibrium dissociation constant for the formation of the heme–hemoglobin complex (6.2 μM) by means of stopped-flow spectroscopy.[81] Spectroscopic and kinetic evidence obtained from other studies involving hemoglobin and other hemoproteins support the theory of the different assembly phases.[88–91] Thus, this model has become widely accepted as the general reconstitution pathway. Although the rate constants obtained indicate that the interaction of free heme with the apoproteins is fast [with the half-life of phase I (heme insertion into apohemoglobin) about 10 ms and that of phase II (rearrangement of polypeptide chains) about 40 s[88]], the complete process that leads to the formation of the fully active holoenzyme can take hours to days. This is likely due to the time needed to achieve the proper equilibration of the heme orientation in phases III and IV. The process also depends to a large extent on the conditions, such as the temperature and pH value. The order of the phases I to IV can also differ from one apoprotein to another. For example, in the reconstitution of apoMb, whose tertiary structure is almost as stable as that of the native Mb (nMb),[90] phase II appears to be missing.[92] On the other hand, in the case of apoHb, 30% of the helical structure elements of native Hb are lost upon heme removal, and all four phases could be observed experimentally during reconstitution.[88]

One of the key questions in these heme-uptake studies was the timing of the heme capture. The investigation of the conformational states of HRP and CCP by means of circular dichroism (CD) and fluorescence spectroscopy during heme removal and re-insertion had indicated that the refolding mechanism of these two peroxidases differs significantly.[93] These studies showed that heme capture in CCP is synchronized with the refolding of the polypeptide chain, while apoHRP captures the heme after the refolding has been completed. Thus, the denatured form of apoHRP does not recognize the heme cofactor and has to fold correctly prior to heme capture. The half-life of the unfolding of HRP is much slower (519 s) than that of CCP (14 s), which indicates that HRP is kinetically more stable that CCP.[93] According to Vasudevan and McDonald, a four-phase mechanism of heme reconstitution also occurs in native HRP, but takes a different order: phases II and III take place first, and are followed by phases I and IV. The study of apoCCP reconstitution indicated that CCP follows the same route as hemoglobin.[93]

A similar mechanism could be envisaged for P450 enzymes, although no data on their reconstitution kinetics have so far been reported. Uvarov et al. reported the presence of five secondary structure forms in native, apo-, and reconstituted holo$P450_{LM2}$ which were estimated from their circular dichroism spectra.[76] It was found that the helix content increased from 34 to 60% upon removal of the heme from the native enzyme, and this change could be reversed by the addition of excess cofactor.[76]

Other enzymes, in particular heavily glycosylated heme proteins, such as chloroperoxidase (CPO), have been found to be notoriously difficult to refold into an active conformation,[94] despite various methods having been tried. In the case of CPO, which is a versatile biocatalyst for many reactions including peroxidative chlorination,[95] dealkylation of heteroatoms,[96] and epoxidation of alkenes,[97] heme is bound to the active site through cysteine ligation.[98] In the course of heterologous expression studies, apoCPO was directly isolated from *E. coli* cells and then reconstituted with native heme. However, under normal conditions only about 1% of holoCPO was obtained and significantly harsher reconstitution methods at high pressure were necessary to increase the amount of active CPO to about 5%, which is to date the best result achieved for this enzyme.[99]

3.2. Reconstitution of Apoenzymes with Non-natural Heme Derivatives

Chemical modification of the heme group can affect one or more of the reconstitution phases described above and it can also be used to alter the function of the hemoproteins. The heme group can be modified in several positions, the most common being positions 2, 4, 6, and 7 of the porphyrin ring (Figure 8) and the central metal ion (replacement with another metal). Of all the heme enzymes, myoglobin has been studied the most extensively as a model protein, and the excellent review articles by Roncone et al.[100] as well as Hayashi and Ogoshi[101] are recommended. Here we will focus on general reconstitution strategies and recent advances concerning a larger variety of heme enzymes.

3.2.1. Structure–Function Relationships

Apoenzyme models have been used to study the structural factors determining the enzyme stability and activity. Several different heme structures are known which mainly differ in their protophorphyrin substituents (Figure 8). These substituents largely affect the activity of enzymes. For example, cytochrome oxidase from *Pseudomonas aeruginosa* shows no oxidase activity at all when its native heme d_1 group (**15**; Figure 8) is replaced with deuteroheme (**19**), mesoheme (**20**), or protoheme (non-iron-containing porphyrin). In contrast, only slight changes in the activity were observed when heme *a* (**11**) was introduced as the cofactor. This result was attributed to the compact structure of these heme groups as well as the lack of a saturated bond between C7 and C8 and the hydroxy group at C2, which affect the enzyme–cofactor interaction.[102]

Numerous purely functional studies were conducted in the 1960s and 1970s, when crystallographic data for a large number of enzymes were not yet available, to probe the interactions between the cofactor and the reaction pocket at the molecular level.[103] More detailed studies of enzymes, such as myoglobin, were later carried out to investigate enzymatic properties as well as to possibly enhance their native activity.[104–111] For example, kinetic studies on the binding of CO to semisynthetic Mb derivatives reconstituted with four different heme groups were performed to gain insights into the effect of the structure on ligand binding. It was found that the association of CO increased up to 20-fold and the dissociation up to 36-fold when the reaction pocket was more accessible through the incorporation of synthetic heme cofactors with smaller molecular volumes.[107] In other studies, the electrochemical properties of reconstituted Mb derivatives were studied with the aim of designing electrochemical biosensors. These studies indicated that changes in the porphyrin ring, such as introduction of nitrogen atoms, influence the electrochemical properties to a significantly lesser extent than changes to the heme environment of the protein scaffold.[112]

The initial heme reconstitution studies also led to scientific interest being focused on the mechanism of heme reorientation in the globin pocket during the in vivo biosynthesis and reconstitution of the native proteins. Various methods were used to elucidate influences of heme orientation on the enzymatic stability and function.[113,114] These methods included the use of modified cofactors containing, for example, fluoride substituents for ^{19}F NMR studies.[115] In another study, Tomlinson and Ferguson investigated protein folding during protein synthesis by replacing two cysteine residues of cytochrome c, which covalently binds heme (heme *c*, **12**), with alanine and reconstituted the mutant apocytochrome *c* with heme *b* (**10**).[116] This led to cytochrome *b* analogues, in which the heme was noncovalently bound. Together with additional structural studies, this observation indicated that the folding of apoprotein occurs prior to heme binding in the process of cytochrome *c* biosynthesis. This result questioned the long standing theory that the cytochromes are exclusively formed by co-translational binding, because the actual requirements for the formation of the holoenzyme in the case of cytochromes *c* and *b* were fulfilled even prior to the heme uptake.[116] More light was shed on the in vivo biosynthesis of holoenzymes by a recent study on Mb reconstitution which showed that that the formation of a compact cofactor–protein structure occurred after the protein was already folded.[117]

3.2.2. Modifications of the Heme Structure

In the search for novel and increased enzymatic activities, the reconstitution of modified cofactors proved to be a powerful tool whose potential remains to be fully explored. Harris et al. exchanged the native heme **10** against two heme derivatives containing different δ-*meso* substituents to investigate the substrate binding and the search for enhanced activity of HRP.[118] This approach enabled the elucidation of an oxidation mechanism for different substrates and led to the identification of a novel HRP which exhibited increased sulfoxidation activity. Previous studies on HRP had shown that heme derivatives with modified carboxy groups at the 6- and/or 7-positions (monomethyl ester and dimethyl ester derivatives) reveal very low or even none of the original peroxidase activity. In contrast, modifications of the vinyl groups at the 2- and 4-positions do not affect the peroxidase activity.[119] This behavior is in contrast to that of modified hemoglobin[120] and myoglobin,[121] where substitutions at positions 2 and 4 strongly affect the O_2 binding properties, whereas the lack of free carboxy groups at positions 6 and 7 does not. DiNello and Dolphin demonstrated that modification of the propionate chains (introduction of an additional methylene group) affected the reconstitution of apoHRP only slightly, but the activity of the resulting HRP was drastically decreased. This finding indicated that not only the presence of the carboxy groups but also their distance from the active site is critical for proper enzymatic activity. Therefore, the size of the heme pocket as well as the distinct interactions of ionized carboxy groups with positive residues on the surface of the protein need to be taken into account when novel enyzmes are designed.[122] Sections 3.2.2.1–3.2.2.4 will focus in more detail on the use of modified heme derivatives to elucidate structure–activity relationships as well as to alter the catalytic properties.

3.2.2.1. Modifications of Positions 6 and 7

The heme propionate groups (positions 6 and 7, Figure 8) of some hemoproteins, such as Mb and cytochrome *b5*, form hydrogen bonds with proximate amino acid residues at the surface of the protein which are believed to stabilize the heme–protein complex.[123] Therefore, reconstitution with heme cofactors containing modified propionate groups can not only significantly alter the mechanism and kinetics of reconstitution but can also affect protein function. Indeed, Hunter et al. reported that elimination of individual hydrogen bonds by site-directed mutagenesis affected the rate at which the heme orientational equilibrium was reached.[124] Moreover, the elimination of these hydrogen bonds decreased the overall thermal stability and led to an increase in the rate constants for heme dissociation. For example, a 10 K decrease in the denaturation temperature of the protein was observed

for cytochrome *b5* when the propionate groups were esterified. Additionally, an about 40-fold increase in the rate constant for heme dissociation was measured for apomoglobin reconstituted with heme containing esterified propionate groups. This change was attributed to an overall destabilization of the heme pocket through the the loss of three hydrogen bonds.[124]

Hayashi et al. reported that the chemical modification of heme propionate chains with eight carboxy groups (**25**; Figure 9) led to alterations in both the substrate specificity and reactivity of Mb, despite the fact that the UV/Vis, CD, and NMR spectra of the modified **rMb25** (r: reconstituted) were comparable to those of native Mb.[125,126] It was also observed that the addition of hydrogen peroxide to reconstituted **rMb25** resulted in the rate of formation of the oxoferryl species (compound II of the peroxidase cycle) increasing more than tenfold compared to that of unmodified Mb.[125] This enhancement indicated that the accessibility of the heme pocket to hydrogen peroxide was improved by structural changes of the heme moiety. Moreover, kinetic measurements under steady-state conditions showed that both the substrate affinity and turnover of **rMb25** for guaiacol were improved, such that the catalytic efficiency k_{cat}/K_M was up to 30 times higher than that of native Mb. In a related study, Mb reconstituted with heme containing four carboxy groups attached to the propionate side chain (instead of eight as in **25**) showed an improvement in the selectivity of O_2 over CO by a factor of 810.[127]

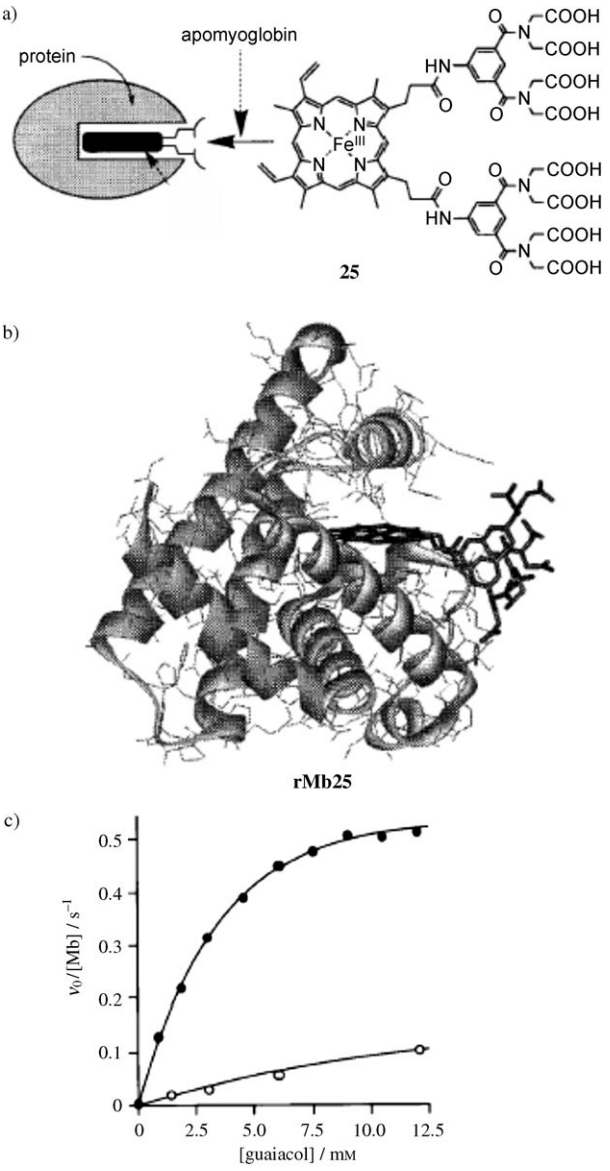


Figure 9. a) Modification of heme propionate groups and the reconstitution of apoMb to **rMb25**. b) Structure of the reconstituted **rMb25** prepared by Hayashi et al.[125] c) An increased activity towards guiacol oxidation was observed with this modified protein (●) relative to native Mb (○); v_0: initial rate. Adapted and reprinted with permission from Ref. [125]. Copyright 1999 American Chemical Society.

The modification of propionate groups can also lead to significant changes in the equilibrium of the heme orientation in reconstituted Mb proteins. For example, Monzani et al. modified Mb by reconstitution with heme in which the propionate group at position 6 was replaced by histidine (Mb-H) or an arginine-alanine dipeptide (Mb-RA).[128] Two different orientations of the heme group are possible in native Mb which differ by a 180° rotation about the α/γ-*meso*-heme axis (**10** in Figure 8). Usually, 92% of the bound heme is in the native and 8% in the 180°-rotated orientation. In contrast, a mixture of four isomers was observed for Mb-H, two with a high-spin and two with a low-spin iron center. However, compared with the native enzyme, the reconstituted Mbs showed only slight changes in the Soret region of the spectra (402–412 nm) and no apparent differences in the visible spectral region. These similarities in the spectra of the modified and native Mb confirmed that local conformational responses were affected by the modification of the propionate groups, but the global conformation was not changed. The esterification can also change the hydrophobicity of the catalytic pocket and thus influence the affinity of the enzyme for hydrophobic substrates. This phenomenon was observed for hemoglobin reconstituted with esterified heme. The resulting enzmye revealed a 30-fold higher affinity for phenolic substrates than did native hemoglobin.[129]

Additional functionalities can be introduced to the propionate side chains of the porphyrin moiety by amide coupling reactions. As an example, phenylboronic acid groups were introduced to the Mb by reconstitution using the modified heme **26** (Figure 10).[130] Phenylboronic acid derivatives can specifically interact with carbohydrates, such as D-fructose, and this interaction can lead to the stabilization of the heme cofactor–apoprotein interaction. Interestingly, this interaction also led to a significant improvement in the oxygen storage capability of the semisynthetic myoglobin **rMb26.**[131] This was one of the first examples which illustrated that the introduction of artificial functional groups at the propionate chains on the heme can be applied for the development of novel biosensors. This concept was further elaborated in subsequent studies.[132,133]

Similar studies on the modification of positions 6 and 7 of the heme are scarce for the P450 enzyme family. However, one example has been reported for the bacterial enzyme $P450_{BM3}$. In this study, the natural heme had been exchanged

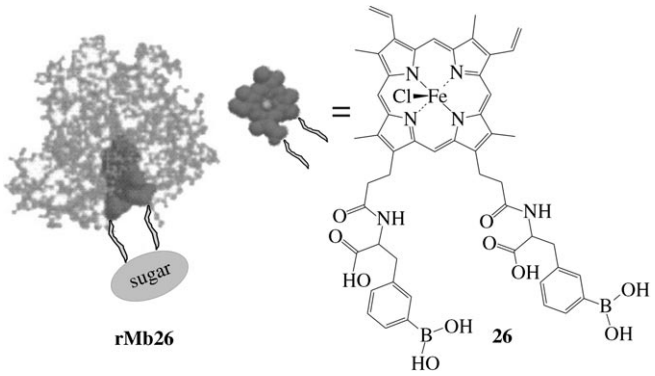


Figure 10. Mb reconstituted with heme containing a phenylboronic acid moiety to enable binding of monosaccharides.[130]

for ferriprotoporphyrin IX dimethyl ester **21** (Figure 8).[74] An enzyme was obtained which had a higher affinity for dodecanoic acid and revealed similar catalytic rates as the native enzyme. Consequently, the reconstituted enzyme had a catalytic efficiency for the hydroxylation of dodecanoic acid that was about threefold greater than its native parent. The enhanced affinity was attributed to an increase in the hydrophobicity of the binding site, because the negative charges of the propionate groups in the vicinity of the fatty acid binding site were removed by esterification. The UV/Vis spectrum of the semisynthetic enzyme was identical to that of the native protein, thus suggesting that the overall structure was not significantly altered.

3.2.2.2. Modifications of Positions 2 and 4

Several studies were undertaken to investigate the effects of modifications introduced directly at positions 2 and 4 of the porphyrin ring. Seybert and Moffat carried out an X-ray crystallography study of horse Hb reconstituted with deuteroheme (**19**) and mesoheme (**20**, Figure 8), which differ in the substituents at their 2- and 4-positions; the products of the reconstitution were **rHb19** and **rHb20**, respectively. Numerous small structural changes in the proximate heme environment of **rHb20** were observed relative to native Hb. In contrast, heme **19** induced only minor and highly localized structural perturbations within **rHb19**.[134,135] Subsequent NMR studies showed that modifications to the side chains attached to the 2- and 4-positions of the porphyrin (as in **20**) can greatly affect the tertiary and quaternary structure of hemoglobin; they also induce changes in the contact regions between the heme and the protein scaffold of **rHb20**, mainly because of distortion of the hydrogen bonds involved.[136,137] In an enzymatic activity study, La Mar et al. found that the reconstitution of apoHb with unmodified heme resulted in fully functional Hb, while replacement with **19** and **20** led to a decrease of about 25- or 100-fold, respectively, in the stability of the enzyme towards denaturing agents, such as urea.[89]

The substituents at the 2- and 4-positions also greatly affect the conformational stability of the heme inside its binding pocket. ^{1}H NMR spectroscopic investigation of heme rotation in sperm whale myoglobin showed that the equilibrium ratio between native (90%) and disordered (10%) heme orientation remains almost unchanged, while the rate of conversion from one configuration into the other depends critically on the substituents at positions 2 and 4. The lowest rates were observed for native heme (**10**; 2,4-divinyl substituted), the fastest for deuteroheme (**19**; 2, 4-dihydrogen substituted), and intermediate for mesoheme (**20**; 2,4-diethyl substituted).[138]

Catalytic studies on reconstituted HRP derivatives suggested that the substituents in the 2- and 4-positions interact sterically with the protein scaffold.[122] However, these sites seem to exhibit a high degree of conformational flexibility, since a vast number of 2,4-disubstituted heme derivatives could be accommodated in apoHRP. The catalytic activity of the resulting HRP derivatives differed greatly, depending on the particular heme modification. For example, HRP modified with **19** and **20** showed 75% and 35%, respectively, of the peroxidase activity of the unmodified enzyme (100%), while HRP modified with 2,4-diformylheme (**22**) showed 60% of the activity. Replacement of the native heme with **20** in cytochrome $P450_{BM3}$ yielded a protein with an unchanged affinity for dodecanoic acid, but a reduced catalytic turnover.[74] This result suggested that the structure of the heme moiety plays a role in the formation of the substrate binding pocket and that variation of the electron density of the heme iron center affects the catalytic properties. Exchange of the vinyl groups of native heme (**10**) with ethyl groups, as in **20**, leads to an increase in the electron density at the heme iron center. Apparently, this leads to a decrease in the rate of heme reduction and, as a consequence, to a decrease in substrate hydroxylation.

3.2.2.3. Modification of Position 8

Position 8 of the porphyrin ring has also been modified, and the resulting heme derivatives have been used to reconstitute peroxidases. Harris et al. reported that the spectra of "compounds I" and "II", which are important intermediates of the peroxidase catalytic cycle,[139] were identical for HRP containing native heme (**10**), 8-hydroxymethylheme (**23**, product: **rHRP23**), or 8-formylheme (**24**, product: **rHRP24**).[140] The rate of formation of "compound I" was the same for native and **rHRP23**, while "compound I" for **rHRP24** was significantly less stable than that of native HRP, and it could only be detected as a transient species. The enzyme **rHRP23** catalyzed the oxidation of guaiacol, iodide, and thioanisole at the same rate as the native enzyme. In contrast, **rHRP24** oxidized the same substrates at lower rates. In particular, an up to four times slower oxidation of guaiacol was observed.[117,118] These results indicate that changes in position 8 can induce alterations in the enzymatic activity as well as of the stability of intermediate compounds in the peroxidase cycle.

3.2.2.4. Replacement of the Iron Center

The heme iron center has been replaced by other metals, such as Co, Zn, and Mn to gain insight into the structure of the active enzymes and intermediates by using different spectro-

scopic methods. For example, cobalt–porphyrin analogues, which are readily obtained by the complexation of Co salts, such as $CoCl_2$ or $Co(OAc)_2$, with the porphyrin ligand, have been used for the reconstitution of Mb and Hb,[141–144] HRP,[145] and P450cam.[75] Cobalt species are versatile probes for electron paramagnetic resonance (EPR) spectroscopy, and such studies enabled detailed investigation of the binding of molecular oxygen and other substrates to the artificial cofactor. In addition, Co and Mn derivatives were utilized to obtain reconstituted myoglobin, which was then extensively investigated by electrochemical means to explore applications in biosensing.[146,147]

3.3. Introducing Novel Functions

The replacement of the native heme cofactor with an artificial derivative containing non-native functional groups opens up the way to generate novel enzymes with altered, enhanced, and even entirely new functions. An overview of representative modifications reported so far and their effects on the activity of the reconstituted enzymes is given in Figure 11. In this section, we will discuss selected examples of such artificial heme enzymes and their applications.

3.3.1. Design of Photoactive Centers

The photoactivation of heme enzymes has been a subject of intensive research because it could provide not only temporal control over the activation of the enzyme but it could also eliminate the need for oxidative activators (such as

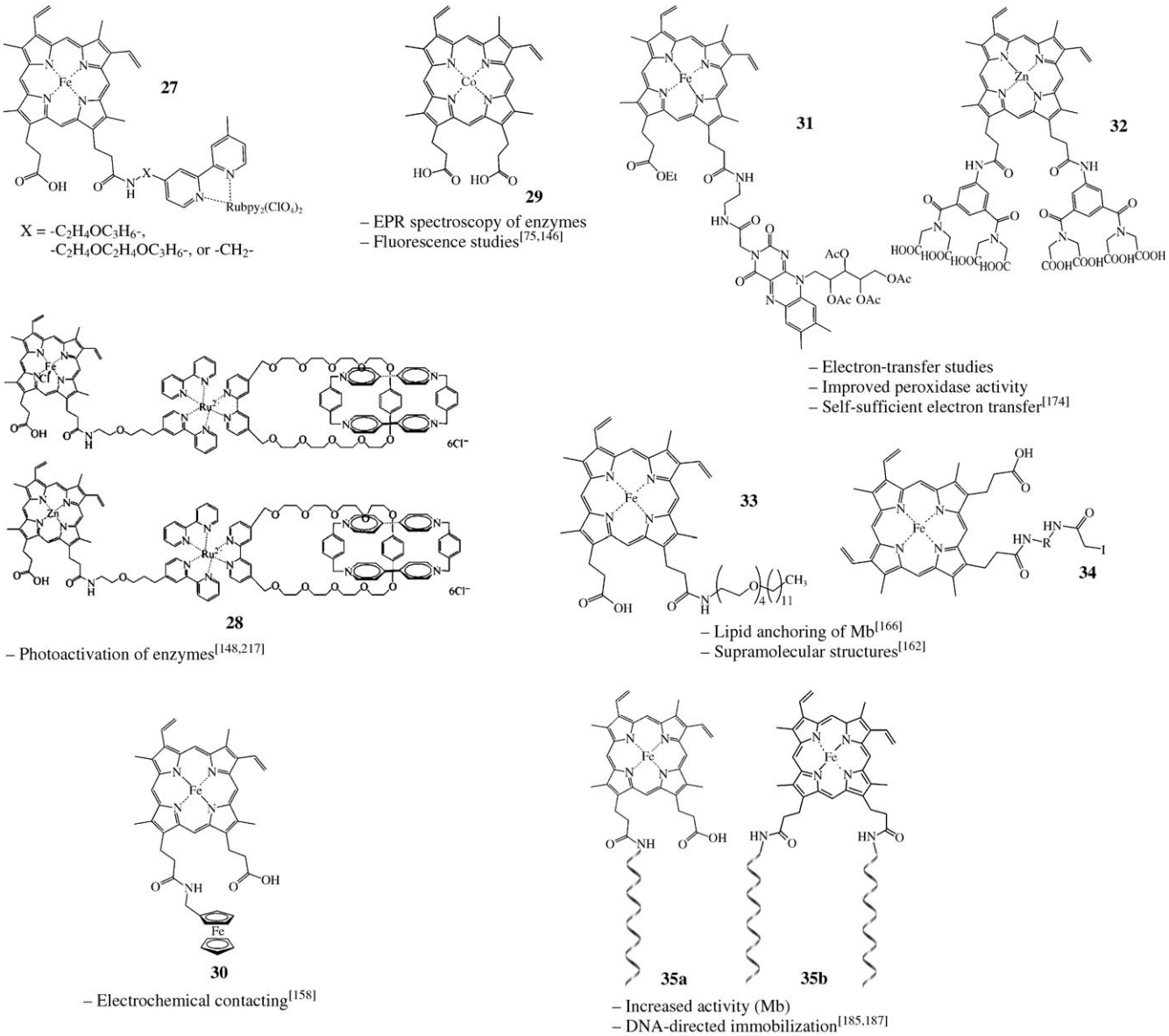


Figure 11. Modified heme cofactors and applications of the respective reconstituted enzymes; bpy: 2,2′-bipyridine.

H_2O_2 for peroxidases or NAD(P)H for oxygenases). To achieve this challenging goal the native enzymes need to be modified with photoactive groups, which can harvest light, harness the energy for redox reactions, and transfer generated electrons to the heme metal center. The native heme cofactor containing two carboxylic acid groups is an excellent starting point for a range of modifications by using site-selective and relatively simple chemical procedures. Much of the seminal work concerning the introduction of photoactivatable groups into myoglobin by reconstitution with artificial cofactors was carried out by the research groups of Hayashi and Shinkai. For example, Hamachi et al. synthesised heme derivative **27** (Figure 11), bearing a photosensitive tris(2,2′-bipyridyl)ruthenium(II) $\{Ru(bpy)_3\}^{2+}$ moiety. Complex **27** can be activated effectively by visible light[148] and it was used for the reconstitution of apoMb to yield **rMb27** (Figure 12). The

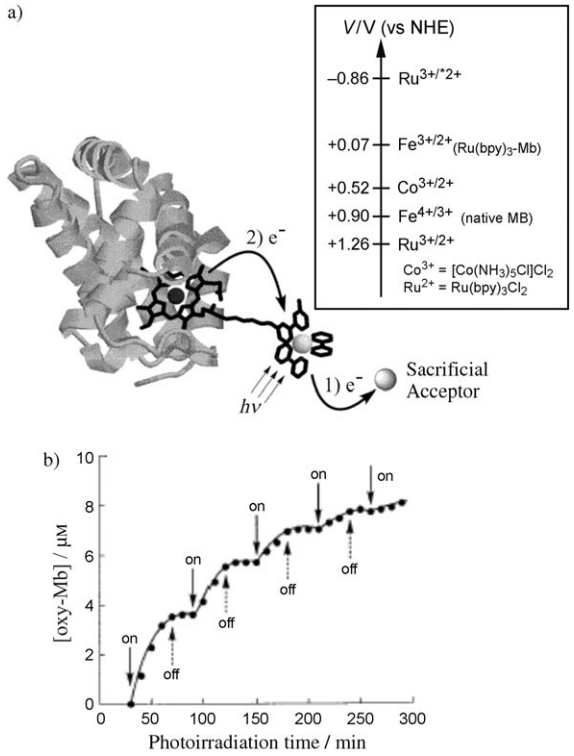


Figure 12. a) Electron abstraction from **rMb27**, which was reconstituted with photoactivatable **27**. Inset: species that take part in the reaction. b) Photochemical production of the dioxygen complex oxy-Mb from **rMb27**; on/off: on and off switching of the visible light. oxy-Mb is only produced on irradiation. Adapted and reprinted from Refs. [148, 150]. Copyright 1993–1999 American Chemical Society.

enzyme-bound cofactor can be reduced from the ferric (Fe^{III}) to the ferrous (Fe^{II}) state, by taking advantage of photoinduced electron transfer, thereby enabling coordination of molecular oxygen at the Fe^{2+} ion. Although the activation with light was only possible when ethylenediaminediacetate (EDTA) was present in high concentrations to act as a sacrificial electron donor, the semisynthetic enzyme could be reversibly switched on and off by irradiation with visible light with a wavelength greater than 450 nm (Figure 12).[149]

Artificial photoactive reaction centers have also been developed from protoporphyrin derivatives which contain chromophores to enable a defined donor–acceptor pathway. Following their seminal work in 1993,[148] Hamachi et al. reported that photoexcitation of semisynthetic myoglobin containing the artificial cofactor **27** (Figure 11) generates compound II (oxoferryl state, Fe^{IV}=O) upon irradiation in the presence of sacrificial electron acceptors such as $[Co((NH_3)_5Cl)]Cl_2$.[150] The $\{Ru(bpy)_3\}^{2+}$ moiety in heme **27** was photoexcited by light and subsequently quenched by $[Co(NH_3)_5Cl]^{2+}$, which acts as a sacrificial acceptor, to produce $\{Ru(bpy)_3\}^{3+}$, which is capable of abstracting an electron from the porphyrin ring and eventually leads to the production of compound II. Compound II is a key intermediate in the peroxidase cycle, and the laser photolysis study of **rMb27** demonstrated for the first time that the photogeneration of compound II proceeds via a porphyrin radical cation. The latter was detectable only because of the accelerated *intra*molecular electron transfer. These results allowed the elucidation of the photoactivation mechanism of **rMb27** and the determination of the rate constants for each step of the cycle. The data obtained are valuable not only for elucidation of the intricate mechanism of the enzyme activation, but also for the future design of novel photoactivatable enzymes.

Studies that built on these results focused on the synthesis of heme cofactors containing linkers of different lengths between the ruthenium complex and the heme moiety.[149] Reconstitution of apoMb with these heme derivatives and comparison of the resulting rMbs revealed a distinct dependency of the photoreactivity on the spacer lengths. The shortest linker led to the least efficient transfer, possibly because the short lifetime of the charge separation was insufficient to permit the subsequent reactions. Similar studies with photoactivatable Ru^{2+} species were preformed by Low et al. on the microperoxidase enzyme.[151] However, instead of reconstitution with heme **27**, the experiments were performed by addition of $[Ru^{2+}(bpy)_3]$ and a sacrificial electron acceptor to the solution of the native enzyme. Spectroscopic studies on the *intra*molecular electron abstraction by $[Ru^{3+}(bpy)_3]$ in this system led to identification of the porphyrin cation radical, which had not previously been described for the microperoxidase-8 (MP8) photooxidation cycle.[151]

In related work, Hamachi and co-workers attempted to circumvent the need to add sacrificial electron acceptors to the solution by tethering appropriate groups directly to the heme cofactor.[152] To this end, Mb-based donor-sensitizer-acceptor triads **28** (Figure 11) were synthesized, which contained heme or Zn porphyrin, an electron acceptor group [cyclobis(paraquat-*p*-phenylene)], and the sensitizer $\{Ru^{2+}(bpy)_3\}$. The latter two moieties were noncovalently linked in a catenane-type fashion, while the $\{Ru^{2+}(bpy)_3\}$ sensitizer was covalently linked to the heme donor. This cofactor was then introduced into myoglobin and the resulting enzyme was studied with respect to the stepwise and vectorial electron transfer. This led to the observation of long-lived charge-separated states upon irradiation.[153] These processes are essential for natural photosynthesis, and thus the design and investigation of such artificial proteins are anticipated to facilitate the development of artificial photosynthetic systems.

Another effective route to the design of photoactivatable biocatalysts by modification of heme with photoactive moieties was developed by Willner et al.[154] They replaced the native iron-containing heme of Mb with Co^{II} porphyrin **29**, and the respective reconstituted Mb was chemically modified with eosine isothiocyanate to yield Eo_2-$MbCo^{II}$ (**rMb29**; Figures 11 and 13). This system was activated by irradiation in

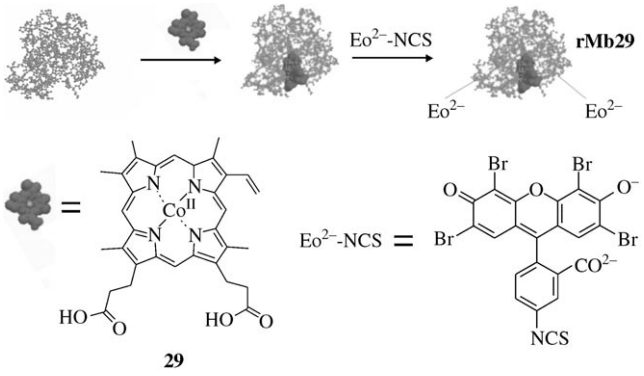


Figure 13. Preparation of photoactivable Mb, which is capable of hydrogenating acetylene.[154]

the presence of Na_2EDTA as the sacrificial electron donor, and it was capable of catalyzing the hydrogenation of acetylene and acetylene dicarboxylic acid. The **rMb29** was also photoactivated in the presence of lactate dehydrogenase and the mediator ferrocene. This resulted in it catalyzing the oxidation of lactic acid and the reduction of acetylene upon illumination ($\lambda = 495$ nm), thus showing its potential to be used as a photocatalyst.[155]

3.3.2. *Electrochemically Active Enzymes*

In the majority of cases, the cofactors of redox enzymes are shielded by the protein scaffold in such way that they are too distant from an electrode transducer to enable direct electron transfer. Small electrochemically active molecules (electron-transfer (ET) mediators) are usually employed in these cases to act as electron shuttles and thus enhance the rate of electron transfer.[156,157] In an attempt to design an electrochemically active enzyme, Ryabov et al. prepared heme–ferrocene conjugate **30** (Figure 11), in which amine-derivatized ferrocene groups were covalently coupled to the propionate chains on the heme.[158] It was known from previous work that HRP reconstituted with heme modified with a monomethyl ester displays only about 20% of the activity of native HRP.[119] Thus, it was questionable whether bulky substituents in these positions of the reconstituted heme enzymes would lead to functional enzymes. Nonetheless, the investigation of the ferrocene-modified **rHRP30** indicated that functional enzymes can be obtained when only one of the propionate groups is modified. Indeed, **rHRP30** showed a threefold decrease in its activity towards the substrate 2,2′-azino-bis(3-ethylbenzthiazolin-6-sulfonate) (ABTS), but its reactivity against artificial organometallic substrates,[159] such as modified ferrocenes, was even higher than that of the native enzyme. The latter increase was attributed to a better accessibility of the reaction pocket of **rHRP30** because of the presence of the modified cofactor. The reaction pocket of **rHRP30** was changed to enable accommodation of nonplanar aromatic substrates, such as ferrocene, more easily than the native HRP. Additional electrochemical studies indicated that the heme-bound ferrocene groups enabled direct electrical communication between the electrode and the heme iron center.[158]

In a related approach, Zimmermann et al. used self-assembled monolayers containing linkers to immobilize heme on gold electrodes and to produce active enzyme electrodes by in situ reconstitution of apoHRP (Figure 14).[160] In this

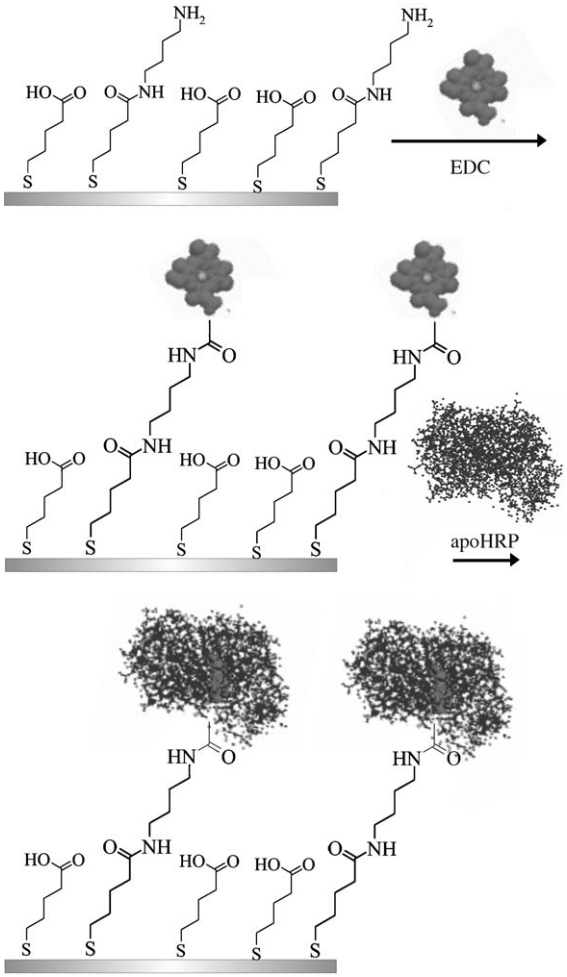


Figure 14. In situ reconstitution of apoHRP on gold electrodes to which heme cofactor was bound through amide coupling by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).[160]

way, both the orientation of the enzyme as well as its distance from the electrode surface can be controlled. The detailed study of reconstituted HRP properties revealed the recovery of the enzymatic activity towards peroxidase substrates.

3.3.3. *Electron-Transfer Models*

Electron-transfer reactions are fundamental to numerous biological processes, such as respiration and photosynthesis.

In natural systems, redox reactions of many heme proteins are often intimately interconnected with reductases, such as cytochromes or flavoenzymes. For example, P450 monooxygenases contain two different domains: one containing the catalytic heme and the other resembling a flavoprotein.[161] Stimulated by these natural examples, Hamachi et al. designed self-sufficient electron-transport systems by reconstituting myoglobin with a riboflavin-modified heme (**31**, Figure 11).[162] When NADH was added as an electron donor to the solution of the modified myoglobin **rMb31** under unaerobic conditions, the reduction of **rMb31** occurred and deoxy-**rMb31** was generated, as monitored by UV/Vis spectroscopy. The reduction rate was enhanced by a factor of 13 compared to the intermolecular system comprised of native Mb and flavin alone. This finding suggested that covalently attached flavin facilitates the electron uptake from NADH.

In many natural systems, electron-transfer reactions between proteins depend on the specific interaction between the two redox protein partners, which, as in many protein–protein interactions, is governed by steric and electronic compatibility of the binding partners. To mimic and to elaborate such processes for artificial ET reactions, Hitomi et al. designed an artificial protein interface using Mb as the model. They used heme **25** (Figure 9) to introduce negative charges on to the Mb surface and replaced the iron with a zinc ion to yield **32** (Figures 11 and 15).[163] The appended carboxylate groups meant that the resulting **rMb32** was able to mimic the natural recognition site of cytochrome *c*. Thus, stable protein–protein complexes with cytochrome were formed, as determined by measurements of the ET rates and binding constants. Further examples of artificial electron transfer model systems based on modified porphyrin derivatives have been comprehensively surveyed in the excellent review by Hayashi and Ogoshi.[101]

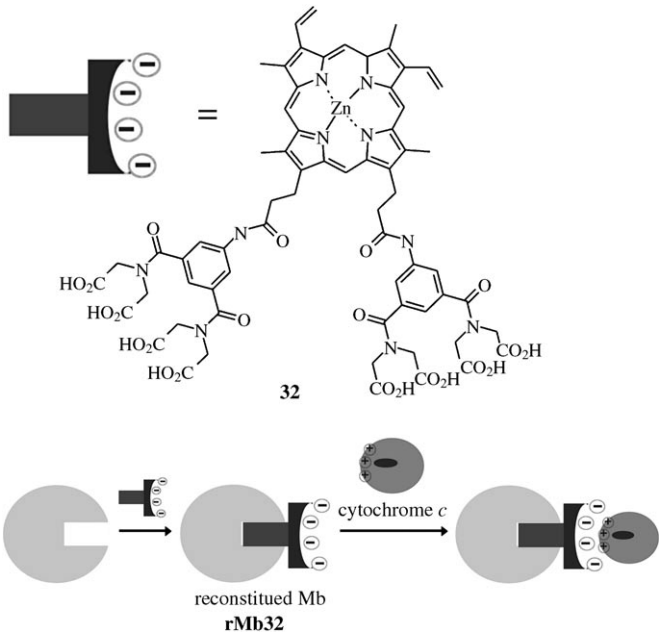


Figure 15. Reconstituted Mb bearing a cytochrome *c* receptor and the formation of a protein–protein complex with cytochrome *c*. Adapted from Ref. [163].

3.3.4. Novel Biomaterials

Increasing interest from the scientific community is nowadays focused on the development of biomaterials which might be used to specifically connect and interact with complex biological systems, such as cells and tissues. Applications of such materials as drugs, drug carriers, biological models, or scaffolds for tissue engineering can be foreseen.[164,165] The use of proteins for the design of novel biomaterials is interesting and challenging from several points of view, primarily because of their biological compatibility and also because of their enormous range of specific functionalities. In recent years, the reconstitution of heme enzymes has been applied to generate novel hybrid materials, and we will present some representative examples and concepts from this area.

3.3.4.1. Lipid-Anchored Myoglobin

Reconstitution of apoMb with long-chain monoalkylated heme **33** (Figure 11) was carried out to produce a synthetic enzyme, which could be anchored specifically to phospholipid bilayer membranes.[166] The absorption, EPR, and CD spectra of this modified Mb indicated that the artificial cofactor was correctly inserted into the active site pocket of apoMb. Gel filtration and ultrafiltration analyses were used to confirm that the reconstituted Mb was bound to lipid bilayer membranes composed of dipalmitoylphosphatidylcholine in an aqueous dispersion. Native Mb revealed no affinity to bind to such membranes. These results, together with the observation that the lipid-anchored Mb was attached to the surface of the lipid bilayer in a fixed orientation, nicely shows how the attachment of an anchor chain to the heme group of Mb can be used as a means to assemble more complex superstructures, thus giving rise to the development of well-defined bionanomaterials.[166]

3.3.4.2. Biohybrid Surfactants

The aforementioned strategy was recently adopted by Boerakker et al. to construct enzyme-functionalized giant amphiphiles.[167,168] To this end, heme modified with polystyrene (PS) was used for the reconstitution of apoHRP and apoMb, and led to semisynthetic enzymes which carry a long hydrophobic tail in an oriented fashion. In aqueous solutions, the PS–enzyme conjugates self-assemble to form biohybrid superstructures, so-called giant amphiphiles. In these giant amphiphiles, the HRP or Mb forms the polar head groups while the synthetic polymer acts as the nonpolar tails, which mediate the formation of spherical aggregates in aqueous solutions (Figure 16a). Polystyrene chains end-capped with a carboxylic acid group were coupled to one of the heme propionate groups to synthesize the amphiphillic monomers. A linker chain was used to span the distance between the carboxylic acid moiety of the cofactor in the active site and the surface of the enzyme. Electron micrographs of the giant

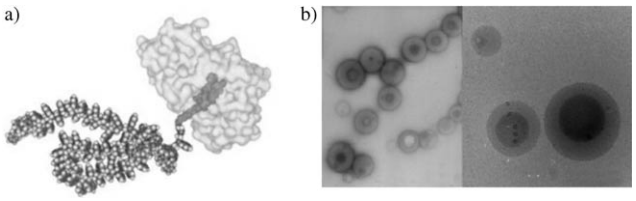


Figure 16. a) Giant amphiphiles prepared by reconstitution of apoHRP (HRP is shown in gray); b) the vesicular aggregates formed. Adapted from Ref. [168].

vesicles revealed that hollow vesicular aggregates with diameters of 80–400 nm were formed (Figure 16b). It was also demonstrated that both HRP and Mb retained their activity, although it was slightly decreased compared to the native enzymes. This study hints towards future approaches in which the assembly of various enzymes and organic catalysts into catalytically active supramolecular structures might lead to new routes for the bioengineering of "artificial cells" and other functional devices.

3.3.4.3. ***Supramolecular Polymers***

Supramolecular polymers are materials made from monomeric precursors through noncovalent interactions at thermodynamic equilibrium. These polymers have attracted the attention of many research groups because they represent a new class of functional and responsive materials.[169–173] In the study by Kitagishi et al.[174] unique submicrometer-sized superstructures of protein-based supramolecular polymers were prepared by using hemoproteins as building blocks (Figure 17). To this end, a cysteine residue was introduced at the surface of cytochrome b_{562} by mutation (**H63CCytb** in Figure 17), and it was subsequently coupled with a chemically activated heme group (**34**, Figures 11 and 17). This yielded a protein with a surface-attached heme (**34holoH63Ccytb**). The native heme cofactor of **34holoH63Ccytb** was then removed by Teale's method and the resulting **34apoH63Ccytb** was allowed to undergo reconstitution with heme groups attached to the surface of the other apocytochrome molecules present in solution. Atomic force microscopy (AFM) studies in the tapping mode showed this led to the formation of large linear assemblies containing more than 100 protein monomers. This approach might be used for the preparation of well-ordered hemoprotein arrays with various biological functions.

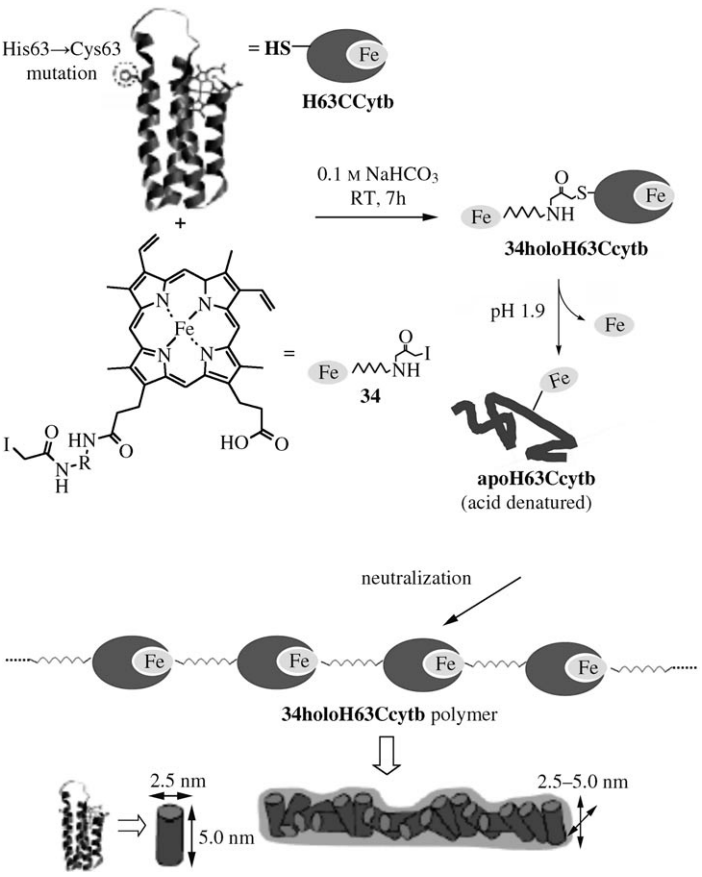


Figure 17. Supramolecular polymers obtained by heme reconstitution of mutant cytochrome b_{562}. Adapted and reprinted with permission from Ref. [174]. Copyright 2007 American Chemical Society.

3.3.5. ***DNA-Modified Enzymes***

As a consequence of their tremendous molecular recognition capabilities, DNA oligomers can be used efficiently as structure-directing agents in the bottom-up fabrication of nanostructured functional devices from protein building blocks.[175,176] The generation of semisynthetic DNA–protein conjugates makes it possible to combine the unique properties of DNA with the almost unlimited variety of functional protein components. However, a great challenge concerns the development of synthetic strategies that permit the control over both the stoichiometry and regioselectivity of DNA–protein coupling reactions. Various approaches have been reported to achieve control over the coupling reactions.[177–184]

A versatile method for the preparation of stoichiometric and constitutionally well-defined protein–DNA conjugates for use in creating protein nanostructures is based on cofactor reconstitution. In a demonstration of this concept, we synthesized heme–DNA conjugates (**35a** and **35b**, Figure 18a), which were then used for the reconstitution of apoMb.[185] The resulting semisynthetic DNA–enzyme conjugates were found to be fully functional and, as a consequence of the appended DNA moiety, capable of specific hybridization to complementary nucleic acids immobilized on a range of surfaces (Figure 18b).[186,187] The DNA–Mb conjugates **rMb35a** and **rMb35b** with heme containing either one or two single-stranded oligonucleotides, respectively, revealed an unexpectedly high peroxidase activity that was significantly higher than native Mb.[185] This phenomenon was attributed to the electrostatic and steric effects of the bulky charged DNA groups, which may induce an opening of the active site of the protein.[186] A similar principle was later used by Sakamoto and Kudo, who coupled 24-mer peptides to heme and used the resulting conjugates for the reconstitution of apoMb.[188] They observed that the heme environment and three-dimensional structure of the resulting rMbs were very similar to those of native Mb, but they also observed an

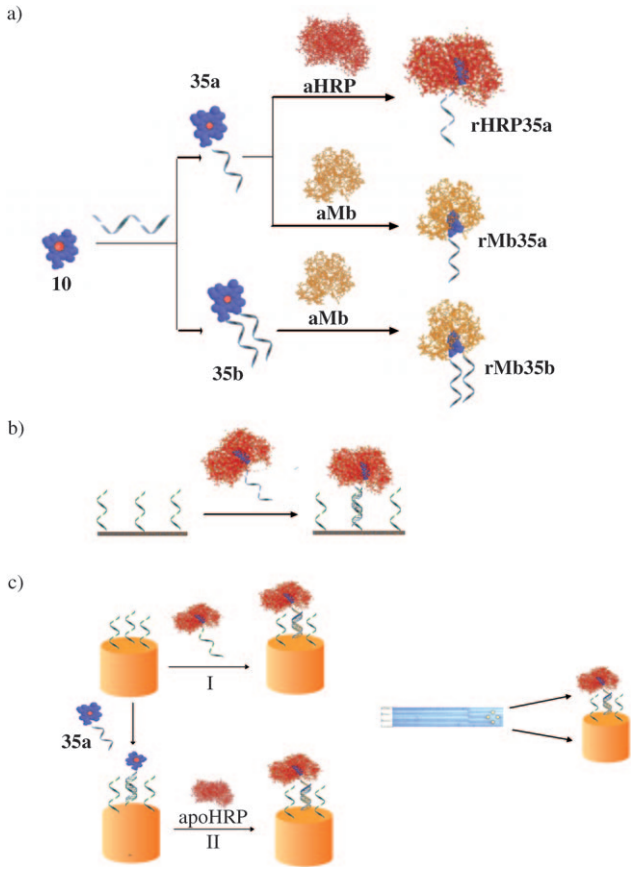


Figure 18. a) Reconstitution of DNA-modified heme into apoMb and apoHRP and b) the subsequent use of DNA to immobilize the enzymes on surfaces. Adapted from Ref. [186]. c) Generation of an array of DNA–HRP conjugates on a Au microelectrode using **rHRP35a** (I) or in situ reconstitution of apoHRP (II). The microelectrode chip containing four gold electrodes is also shown. Adapted from Ref. [187].

enhancement in the peroxidase activity, similar to the results observed in the case of the DNA-modified Mb **rMb35**.[186]

The reconstitution of apoenzymes with heme–DNA conjugates was also used to generate arrays of DNA–HRP conjugates on microelectrode surfaces by taking advantage of DNA-directed immobilization (DDI) method (Figure 18c).[187] The use of the redox mediator *ortho*-phenylendiamine and hydrogen peroxide enabled measurement of the amperometric response of the DNA-immobilized HRP. Such arrays of redox enzymes might be useful for the screening of drugs or the detection of environmental pollutants or warfare agents. The DNA-directed immobilization of **35a** was used very recently in conjunction with a surface plasmon resonance biosensor to quantitatively determine the kinetic rate constants of heme uptake and the dissociation of apoenzymes.[189]

3.3.6. *Insertion of Novel Cofactors*

In the examples given in Sections 3.3.1–3.3.5, the exchange of the naturally occurring heme for a range of modified porphyrin derivates enabled the introduction of a number of novel functions into the enzymes. It has also been demonstrated that the heme cofactor can be replaced by artificial heterocyclic compounds of similar size. One impressive example is illustrated by the use of iron porphycene **36** (Figure 19) as an artificial prosthetic group to obtain a

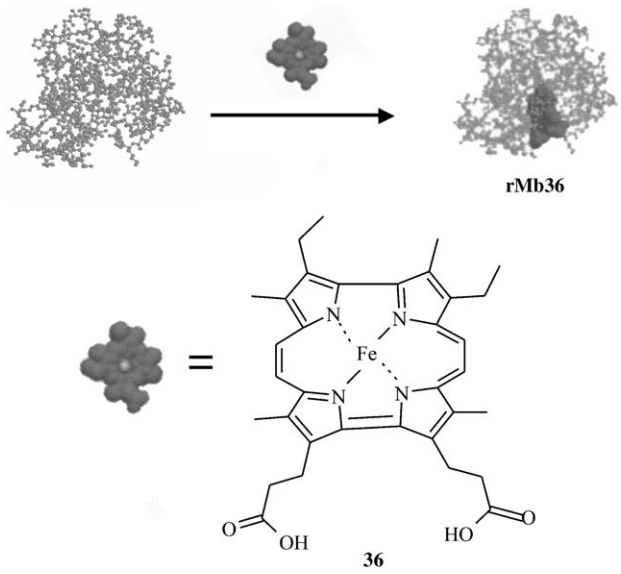


Figure 19. Preparation of **rMb36** by reconstitution of apoMb with iron porphycene **36** as an abiotic cofactor. **rMb36** has a blue color and displays strongly increased oxygen affinity.[190]

reconstituted Mb derivative.[190] The replacement of heme with the porphycene group induced a marked change in the color of the resulting enzyme: **rMb36** is blue, while native Mb has a brownish red color. The **rMb36** also showed a change in its O_2 binding properties: The association rate of O_2 was increased 5-fold while the dissociation rate was 250 times lower than that of native Mb. Thus, determination of the binding kinetics revealed **rMb36** had a significant, about 1400-fold, higher O_2 affinity which is as high as that of the native O_2-storage protein haemoglobin.

In an attempt to design novel metalloenzymes, Ohashi et al., exchanged heme for a cofactor of entirely different structure.[191] It is known that chromium-containing Schiff base complexes catalyze various oxidations in organic solvents. It was investigated whether chromium-containing salophen ligands could be used to replace the native cofactor and thus create novel catalysts. Taking into account several conformational aspects, such as potential contact points between the Schiff base and the protein, a mutant Mb was generated and reconstituted with chromium(III)–salophen **37** (Figure 20). Indeed, the resulting semisynthetic enzyme **rMb37** was capable of catalyzing the H_2O_2-dependent sulfoxidation of thioanisole.[191] Although the enzyme exhibited low reactivity and enantioselectivity, this study was a clear demonstration that artificial metalloenzymes can be obtained through the combination of protein engineering and coordination chemistry. Subsequent reports on the X-ray crystal structures of the apoMb mutant with chromium and manganese Schiff base ligands enabled detailed insights to be

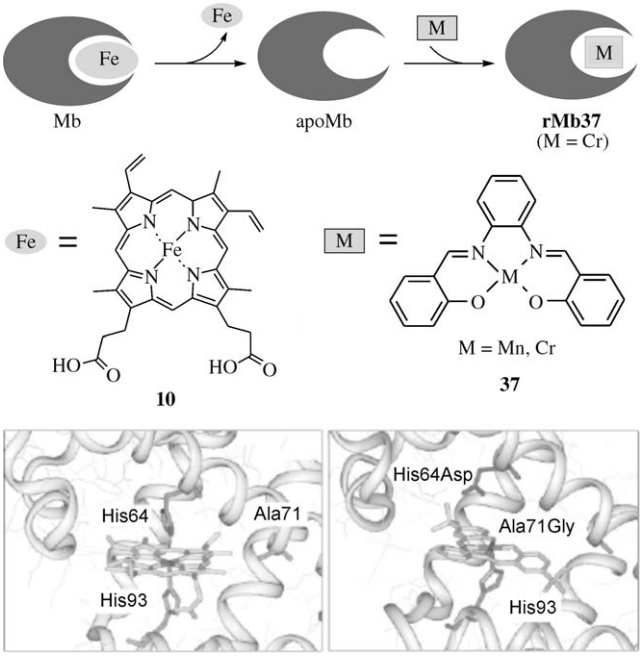


Figure 20. Top: Preparation of artificial metalloenzymes by reconstitution of apoMb with Mn- or Cr-containing salophen. Bottom: molecular models of the active centers of the native (left) and artificial Mb (right). Adapted from Ref. [191].

made into the interactions of the artificial cofactor with the enzyme.[192] In addition, the enantioselectivity of the thioanisole sulfoxidation was improved by changing the size of the salophen (**37** in Figure 20) substituents.

In similar approaches, non-native prosthetic groups such as Mn^{III}, Fe^{III}, and Cr^{III} Schiff base complexes were inserted into carefully designed apoMb, where amino acids had been exchanged (for example, Ala71 to Gly) to enable tighter binding to the reaction pocket.[193] Lu and co-workers incorporated manganese–salen complexes (salen = *N*,*N*′-bis-(salicylidene)ethylenediamine) into a mutant apoMb (Leu72Cys-Tyr103Cys) through reaction of engineered cysteine residues with the methane thiosulfonate group of the manganese complex.[194] The procedure allowed the covalent linkage of the artificial cofactor in the heme pocket after insertion, with a high amount of control over its orientation within the protein scaffold. This led to semisynthetic enzymes which revealed increased rate constants for the enantioselective sulfoxidation (from 0.078 to 390 min^{-1}) and greater enantionselectivity (increase from 13 to 51 % *ee*) compared to the noncovalent strategy.[191] This study also emphasizes the feasibility of inserting synthetic metal complexes into designed reaction pockets of selected enzymes to tailor the selectivity and efficiency of novel biocatalysts.[195]

4. Other Cofactors—Reconstitution of Pyrroloquinoline Quinone (PQQ)

Pyrroloquinoline quinone (PQQ, **38**; Figure 21) was first described in 1979 as a cofactor of bacterial alcohol dehydrogenases.[196] This cofactor was recently recognized, together

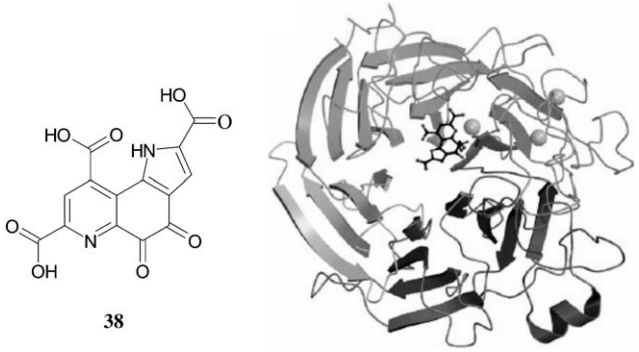


Figure 21. The structure of cofactor PQQ (left) and PQQ-containing enzyme glucose dehydrogenase (right). In the enzyme structure, PQQ is shown as a black framework and the Ca^{2+} ions necessary for PQQ coordination are depicted as gray spheres.

with riboflavin, as one of the B vitamins. It plays an important role in metabolic conversions, particularly as a cofactor of various enzymes, such as 2-aminoadipic-6-semialdehyde dehydrogenase (AASDH) which is involved in the degradation of lysine.[197] A number of other quinone cofactors has since been described.[198] The enzymes containing PQQ—quinoproteins, copper quinoproteins, and quinohemoproteins—are mainly involved in the direct oxidation of alcohols, sugars, and amines.[199]

PQQ acts as a cofactor and a redox shuttle. Furthermore, PQQ enzymes have been used in the design of electrical biosensors since they often permit direct electron transfer between the electrode and the enzyme.[200] To this end, PQQ enzymes have been immobilized directly on carbon electrodes[201] or conducting polymers,[202] and they were employed in the development of glucose[203] and ethanol[204] sensors. The natural electron acceptor for PQQ dehydogenases is usually not dioxygen but rather ubiquinones and cytochromes, which are present in the cell.[205] There are no covalent bonds involved in the binding of PQQ to apoenzymes, but the cofactor is coordinated through Ca^{2+} [206] or Mg^{2+} ions.[207] It is, therefore, possible to readily remove PQQ from the enzymes and then reconstitute the apoenzymes with artificial cofactors so as to investigate the structural and catalytic properties of PQQ proteins.

As a consequence of its electron-shuttle function, PQQ was used in the design of enzyme electrodes, similar to that described for FAD in Section 2.3. In one of the first examples of the use of PQQ for the development of electrochemical sensors, Katz et al. immobilized PQQ through amide coupling with appropriate linker groups on a gold electrode.[208] Such modified electrodes were then employed for the in situ reconstitution of apoglucose dehydrogenase (apoGDH), which was prepared by denaturation of the native enzyme and removal of the cofactor by gel filtration. The initial measurements of the electrochemical response in the presence of glucose as the GDH substrate suggested that almost no direct communication occurred between the reconstituted GDH and the gold electrodes. When 2,4-dichlorophenolindophenol (DCPIP) was added as an electrochemical mediator, however, an electrochemical response was detected. This finding suggested that the enzyme is active, but no direct

communication is possible due to the insulating protein shell. This problem was later circumvented by the employment of more efficient linker systems. For example, electrically contacted GDH enzyme electrodes were successfully constructed by the reconstitution of apoGDH on PQQ-functionalized polyaniline films.[209] In this approach, PQQ was coupled to a polyaniline/polyacrylic acid composite film deposited on a gold electrode through amide coupling. Such electrodes were used for the in situ reconstitution of apoGDH to yield sensors capable of detecting the biolectrocatalyzed oxidation of glucose. This system was later improved by using gold-nanoparticle relays (Figure 22) similar to those used for

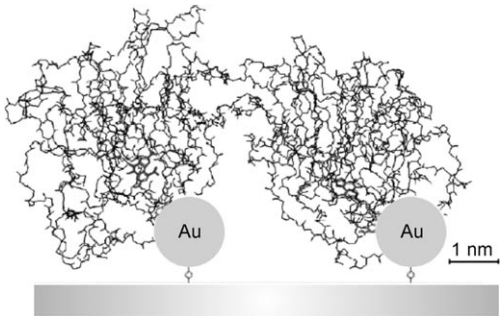


Figure 22. Immobilization of apoGDH onto the PQQ-modified gold nanoparticles attached to a gold electrode. Adapted and reprinted with permission from Ref. [206]. Copyright 2005 American Chemical Society.

the electrical contact of the FAD-dependent enzymes (Figure 7). The use of the PQQ-modified gold nanoparticles for the functionalization of gold electrodes resulted in a 25-fold improvement in the electrical contact with GDH, as compared to the analogous electrodes with polyaniline films.[206]

The reconstitution of PQQ enzymes was also utilized to investigate the structure–function relationships and the molecular mechanisms of the catalytic activity. By far the most extensively studied enzyme of the PQQ family is the soluble GDH from *Acinetobacter calcoaceticus*.[210] The second member of the GDH group, membrane-bound GDH (mGDH),[211] has also been studied extensively. It was long believed that this enzyme undergoes the same mechanism of action as the soluble form. Interestingly, the reconstitution-based activity studies with apomGDH expressed from *E. coli* revealed that those two enzymes differ significantly:[212] It was discovered that mGDH binds PQQ less strongly than does soluble GDH and that, contrary to soluble GDH which requires Ca^{2+} ions for complete reconstitution, mGDH requires Mg^{2+} ions. Moreover, unlike soluble GDH, the reduced mGDH reacts with O_2, and this unexpected phenomenon is still under investigation.

Iswantini et al. monitored the formation of holo mGDH in vivo after the addition of PQQ and glucose by using *E. coli* deposited on the surface of a carbon paste electrode.[213] Electrochemical studies were used to determine the equilibrium constant for the PQQ reconstitution in the cell as well as to assess the activity of reconstituted enzymes.

Despite this progress, it should be noted that the studies on PQQ reconstitution are still in its infancy, in particular, when compared to FAD- and heme-related investigations. PQQ reconstitution has mainly focused on the design of electrochemical biosensors and it can be foreseen that future work will also aim at the use of modified PQQ to generate novel functional proteins for other applications, such as novel biomaterials.

5. Summary and Outlook

The reconstitution of flavo, heme, and PQQ enzymes with native and artificial cofactors has been proven to be an effective method for investigating the structural and functional properties of enzymes. Moreover, reconstitution of the apoenzyme can be used as a powerful tool for the introduction of novel functionalities into natural protein scaffolds, and gives rise to new hybrid devices and materials. One particular advantage of this approach stems from the almost unlimited possibility to engineer tailor-made functional groups into natural cofactors by using state-of-the-art synthetic chemical methods. The scope of this approach is further enhanced by the steady improvement of methods to create modified protein scaffolds by means of site-directed mutagenesis or in vitro evolution. Taking into account the constant developments of suitable high-throughput screening assays, one may thus anticipate that cofactor reconstitution will open up new routes to the generation of novel biocatalysts. The feasibility of this perspective has already been demonstrated.[190,191] Moreover, in view of the current advances in nanobiotechnology and the efforts to design tailor-made nanoparticles as transducing[57] or co-catalytic[214–216] elements in nanoparticle–biomolecule hybrids, it can be envisaged that the scope of possible applications of cofactor reconstitution will reach far beyond the design of biosensors. Therefore, one may speculate that biocompatible, environmentally responsive, or other functional materials will be accessible through this approach. It is certain, however, that this research at the interface of chemical biology and nanosciences will provide a challenging playground for creative chemists in the next decade.

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[1] In contrast to the cofactors and prosthetic groups discussed here, NAD(P) is bound loosely to the enzymes because of its low affinity to apoenzymes and its transient nature (D. Voet, J. G. Voet, *Biochemistry*, Wiley, Somerset, **1995**). Thus, it actually functions as a diffusable cofactor. Although chemical modifications of NAD have been reported, for example, with ferrocene (T. Kijima, T. Suzuki, T. Izumi, *J. Biosci. Bioeng.* **2003**, *96*, 585) or PEG (K. Okuda, I. Urabe, H. Okada, *Eur. J. Biochem.* **1985**, *151*, 33), such modified cosubstrates were added to the solutions of an already functional enzyme and not to the apoenzyme. The diffusional nature of such cosubstrates means this case is markedly different from the situation of tightly bound prosthetic groups, such as heme, FAD, and PQQ, which are permanently associated with an enzmye.

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