ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 2, 2009

DOI: 10.1089/ars.2008.2098

Comprehensive Invited Review

Protein Engineering of Redox-Active Enzymes

Gloria Saab–Rincón[1] and Brenda Valderrama[2]

Reviewing Editors: Robert Hondal, H. Rex Gaskins, and Andre Palmer

Abstract

Redox-active enzymes perform many key biological reactions. The electron transfer process is complex, not only because of its versatility, but also because of the intricate and delicate modulation exerted by the protein scaffold on the redox properties of the catalytic sites. Nowadays, there is a wealth of information available about the catalytic mechanisms of redox-active enzymes and the time is propitious for the development of projects based on the protein engineering of redox-active enzymes. In this review, we aim to provide an updated account of the available methods used for protein engineering, including both genetic and chemical tools, which are usually reviewed separately. Specific applications to redox-active enzymes are mentioned within each technology, with emphasis on those cases where the generation of novel functionality was pursued. Finally, we focus on two emerging fields in the protein engineering of redox-active enzymes: the construction of novel nucleic acid-based catalysts and the remodeling of intra-molecular electron transfer networks. We consider that the future development of these areas will represent fine examples of the concurrence of chemical and genetic tools. *Antioxid. Redox Signal.* 11, 167–192.

I. Introduction

THE REDOX CHARACTER OF AN ENZYME is a complex trait. It is naturally influenced by the amino acid sequence of the protein due to the presence of redox-active natural residues such as cysteine or tryptophan, although nonencoded residues may also be incorporated by post-translational modification. The primary sequence also encodes for the potential conformation of cofactor binding pockets, whether metallic or organic. At the next level, the native fold pattern

[1]Departamento de Ingeniería Celular y Biocatálisis and [2]Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Cuernavaca, Morelos, México.

of a protein displays a unique distribution of charged and uncharged amino acid side chains on the surface that determines the electrostatic potential surrounding the molecule. Finally, the specificity and efficiency of intra- and intermolecular electron-transfer pathways depend on the interaction of all the previous features.

Redox-active proteins and enzymes carry out many key reactions of biological importance. The underlying process essential for these reactions is electron transfer. In general, oxidative enzymes abstract two electrons from the substrate; the intermediates are stabilized by the active site to avoid the uncontrolled production of reactive species. A subset of the redox enzymes transfer a single electron, in a free radical-based catalysis. In the latter case, the highly reactive intermediates are stabilized as protein-based free radicals. Given the importance of redox-active enzymes, a profound knowledge of their catalytic mechanisms has been accumulated. This knowledge, along with the recent optimization of different protein engineering tools, gave rise to a promising scenario for the development of novel strategies aimed at the generation and modulation of redox properties in enzymes.

II. Basic Features of Redox Sites

A. Amino acid residues

From the 20 genetically encoded amino acids, only cysteine undergoes facile redox chemistry. The functional group of cysteine is the thiol (-SH) moiety, sometimes called the sulfhydryl or mercapto group, which is prone to form disulfide bonds between cysteine residues that are close together in space. The disulfide bridge between cysteine residues can be released by reducing agents that include other thiols. Reaction of cysteine thiols with organic or inorganic oxidants leads to the potential formation of at least seven different oxidation products, including protein-sulfenic, -sulfinic and -sulfonic acid derivatives (Fig. 1). The physiological potential of these post-translational modifications, regarded by protein biochemists in the past to be a result of unspecific oxidative damage, has recently acquired a preeminent role in the field based on novel roles, a selection of which will be described next (103, 167, 201).

Cysteine thiols are sensitive to hydrogen peroxide down to micromolar concentrations and react to form sulfenic acid derivatives. Higher hydrogen peroxide concentrations effectively convert catalytic cysteine-sulfenic acid groups into sulfinic acids, with subsequent loss of enzyme activity (54, 83). Cysteine-sulfenic acids and disulfides are known to be reduced by glutathione or thioredoxin in biological systems, but cysteine-sulfinic acid derivatives have been regarded as irreversible protein modifications. The recent identification of sulfiredoxin, a protein able to reduce cysteine-sulfinic acid in *Saccharomyces cerevisiae*, revealed the exquisite simplicity of the cysteine-based redox sensing circuitry that endows systems with the ability to respond not only to peroxides, but also to superoxide and to reactive nitrogen species (41, 238).

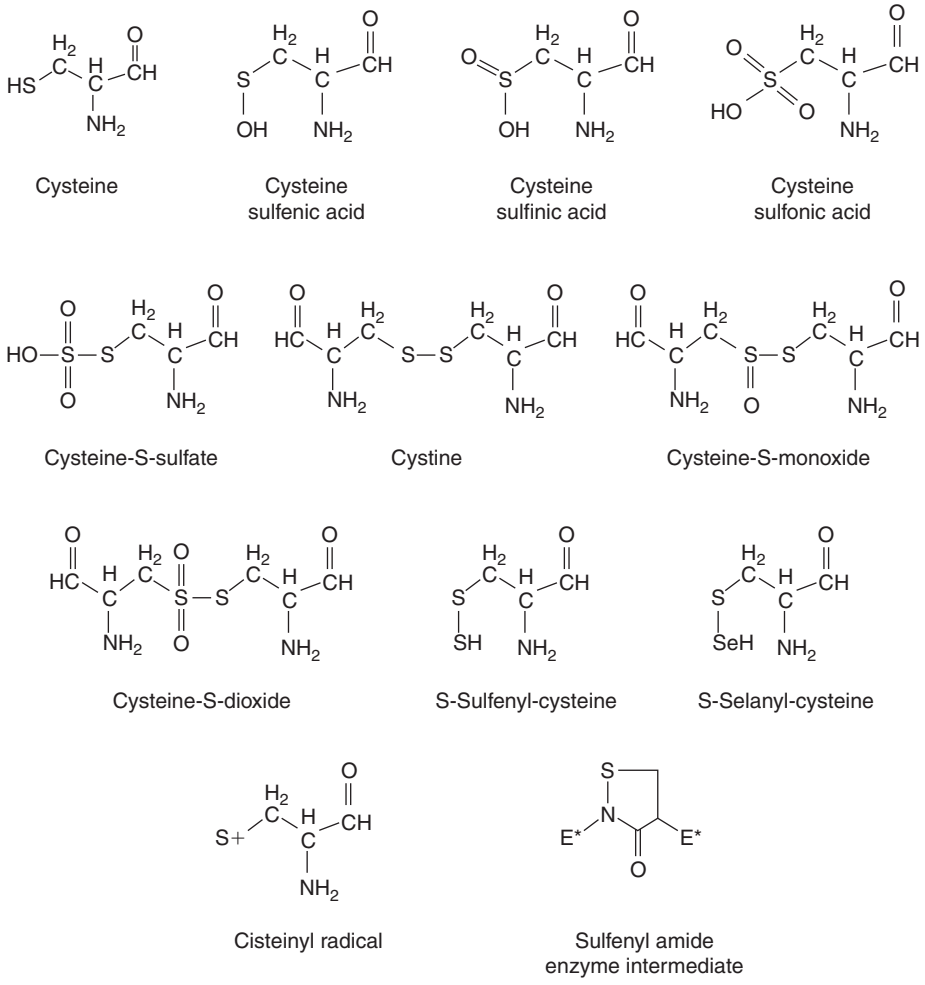

FIG. 1. Common modifications of cysteine confirmed or postulated to occur *in vivo*.

The physiological relevance of this reaction was recently confirmed by the demonstration of how the selective transition between sulfenic and sulfinic acids in thioredoxin peroxidase 1 (Tprx1) of *S. pombe* regulate the hydrogen peroxide-sensing Pap1 pathway (225). An equivalent chemistry has been described in prokaryotes, where the transcriptional regulator OhrR serves both as a sensor and effector of oxidative conditions (157). Furthermore, the formation of a sulphenyl-amide intermediate creates an elegant mechanism to protect the catalytic cysteine residue in the human protein tyrosine phosphatase 1B where the irreversible oxidization to sulfinic or sulfonic acids lead to protein deactivation (222). Taken together, these data indicate that the controlled reduction of cysteine-sulfinic groups serves a cellular function possibly as important as the regulatory and catalytic roles of cysteine-sulfenic residues.

Despite the increasing awareness of the critical roles played by cysteine residues in proteins, the identification of function for specific positions is still the limiting step for protein engineering. So far, relevant positions have been identified case-by-case using both chemical and molecular strategies, including countless reports of site-directed substitution. In recent publications, bioinformatic tools have been applied to the prediction of cysteine residue variations. Fiser and Simon, based on the observation that Cys tends to occur in the same oxidation state within the same protein, predicted the redox state of homologous positions (74). Fomenko and collaborators exploited the natural occurrence of the rare amino acid selenocysteine, previously identified as part of active sites serving thiol/selenol-based redox functions (103). Based on a genome-wide survey of proteins homologous to those where selenocysteine residues have been identified, the authors developed a large-scale procedure for the identification of previously unknown catalytic redox-active cysteine residues in proteins (76, 78) . Interestingly, only cysteine residues involved in thiol-based catalysis were detected by the method, leaving out residues involved in indirect redox-related functions, such as metal binding. The method is simple and highly predictive and will undoubtedly expand our knowledge of physiologically relevant cysteine-based redox enzymes.

B. Quinoproteins

The field of quinoproteins began with the discovery and characterization of a low molecular weight, dissociable cofactor from bacterial alcohol dehydrogenase, designated pyrroloquinoline quinone (PQQ). Since then, it has been clearly demonstrated that nonbound quinones, including ubi-, mena-, and plastoquinone, are essential components of

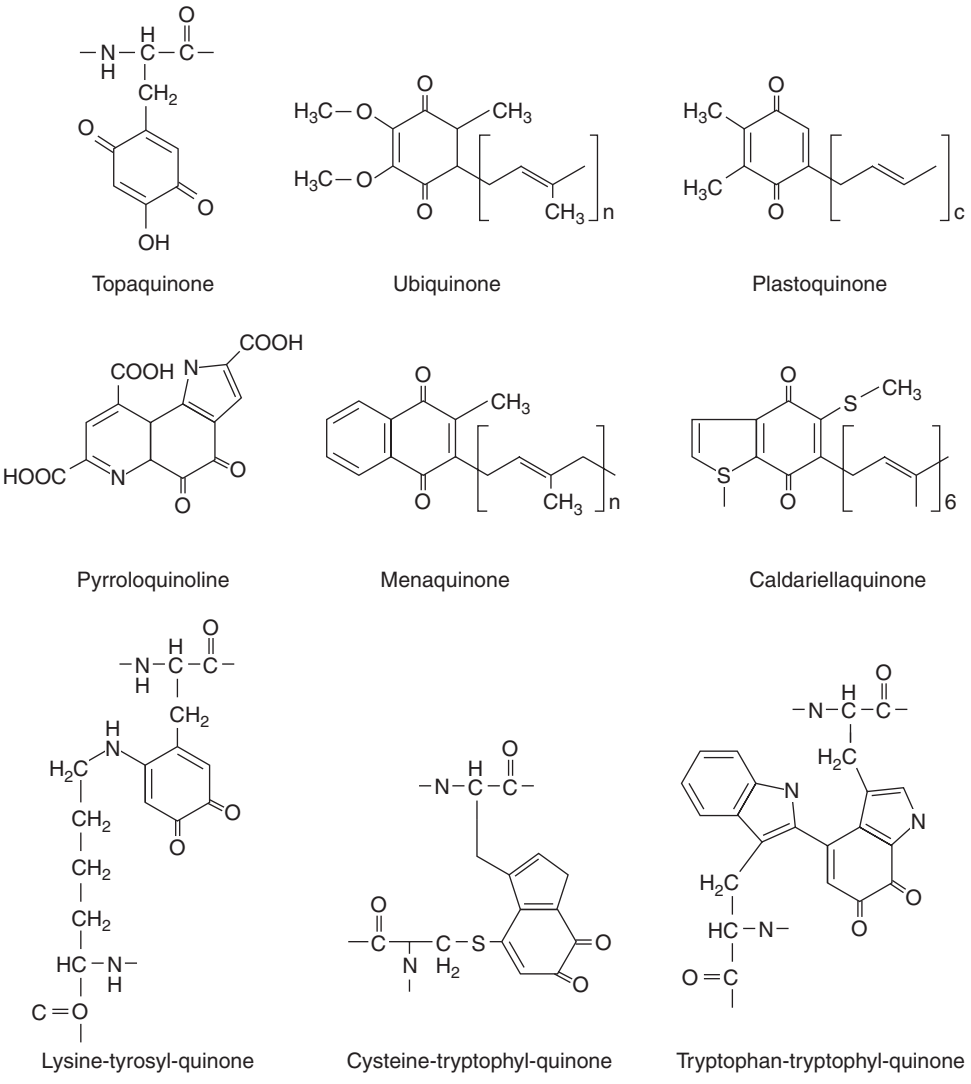

FIG. 2. Structure for quinocofactors.

membrane-bound electron-transfer reactions and for the conversion of redox energy into the proton gradient required for ATP synthesis. As enzyme cofactors, quinones may be present in the form of noncovalently bound elements such as PQQ and caldariella quinone. However, there is also a diversity of covalently-bound quinones, derived from protein modification that also participate as cofactors in redox reactions, such as topaquinone (TPQ), lysine tyrosyl-quinone (LTQ), tryptophan-tryptophyl-quinone (TTQ), and cysteine-tryptophyl-quinone (CTQ) (Fig. 2) (8, 51, 59, 61, 134). PQQ is the prosthetic group of dehydrogenases for methanol, higher alcohols, aldose sugars, aldehydes, and polyvinyl alcohol, and for hydroxylation of lupanine. Amine dehydrogenases may present TTQ, derived from two tryptophan residues, or CTQ, derived from an *o*-quinone-modified tryptophan side chain covalently crosslinked to a cysteine residue, as cofactors (50). Most copper-containing amine oxidases use TPQ, a modified tyrosine residue as cofactor, with the exception of lysyl oxidase that makes use of LTQ. Details of the catalytic mechanism of all these enzymes have been described elsewhere (6, 8, 60, 84, 113). The mechanism of amine oxidation, one of the best characterized for quinoproteins, occurs through the formation of enzyme–substrate covalent adducts with TPQ, TTQ, CTQ, and LTQ redox centers (50, 145, 188, 232). The final electron acceptors of quinoproteins may be other soluble redox enzymes such as *c*-type cytochromes or amicyanin which have been widely studied as model systems for the characterization of intramolecular electron-transfer reactions (7, 52, 53).

C. Flavin-binding proteins

Flavoproteins are enzymes that catalyze redox reactions using flavins as cofactors. The most abundant natural flavins are riboflavin and its two main derivatives, flavin mononucleotide (FMN), with a phosphate group attached to the 5′ carbon atom of the ribose chain, and flavin adenine dinucleotide (FAD), further derived from FMN by addition of a diphospho-adenosine moiety at the 5′ phosphate terminal. The fused ring structure of flavin nucleotides (the isoalloxazine ring) undergoes reversible reduction, accepting either one or two electrons in the form of one or two hydrogen atoms from a reduced substrate, so they can exist in three different redox states, namely the oxidized quinone, the one-electron reduced semiquinone, and the two-electron reduced hydroquinone (Fig. 3). These states are spectroscopically distinct from each other, allowing the progression of electron transfer between the different states to be followed over time.

Because of the stability of the semiquinone form, flavoproteins can participate in either one- or two-electron transfers, allowing them to be involved in a greater diversity of reactions than the pyridine nucleotide-linked dehydrogenases. Thus, a flavoprotein can oxidize a reduced substrate by removal of two electrons and transfer them as a pair to a two-electron acceptor such as molecular oxygen to form hydrogen peroxide, or individually to a one-electron acceptor such as a cytochrome. A common reduced substrate for many flavoproteins is NADH or NADPH serving as a cellular electron source which can then be targeted to specific electron acceptor compounds of proteins by the flavoprotein. The nature of the electron acceptor defines the physiological role of the flavoproteins, whereas the selective transfer of single electrons from one to other proteins defines a pure electron transferase. Coupling to small substrates

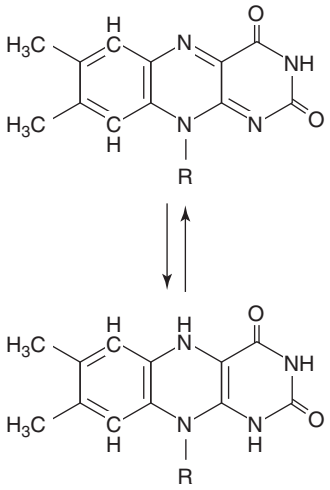

FIG. 3. Oxidized (*upper*) and reduced (*lower*) forms of the isoalloxazine ring of flavin nucleotides. R indicates the remainder of the coenzyme molecule.

further divides them in dehydrogenases/oxidases, dehydrogenases/oxygenases, transhydrogenases, and dehydrogenases/electron transferases

In general, the flavin nucleotide is tightly bound to flavoproteins, and in some cases, it is even covalently bound. In consequence, flavin nucleotides do not transfer electrons by diffusing from one protein to another; rather, they provide a means by which the flavoprotein can temporarily hold electrons while it catalyzes electron transfer from a reduced substrate to an electron acceptor. One important feature of the flavoproteins is the variability of the standard reduction potential of the bound flavin nucleotide. Tight association between the enzyme and the cofactor confers on the flavin ring a reduction potential typical of that particular flavoprotein. Flavoproteins are often very complex; some have, in addition to a flavin nucleotide, tightly bound metal ions (45, 107, 133, 215).

D. Pyridine nucleotide-bound proteins

Nicotinamide adenine dinucleotide (NAD+ in its oxidized form) and its close analog nicotinamide adenine dinucleotide phosphate (NADP+) are composed of two nucleotides joined together through their phosphate groups by a phosphoanhydride bond. Both molecules undergo reversible reduction of the nicotinamide ring by accepting a hydride ion from the oxidized substrate (Fig. 4). The disparate concentration of these two analogs in the cell, such that NAD+/NADH is 10-fold more concentrated than the NADP+/NADPH pair, results in a functional specialization favoring the participation of NAD+ in oxidation reactions and NADPH in reduction reactions. A few enzymes can use either cofactor, but most show a strong preference for one over the other. Currently, >200 different enzymes have been described which are known to catalyze reactions with these molecules as redox cofactors. The association between the protein and NAD or NADP is relatively loose and the cofactor readily diffuses from one enzyme to another, acting as a water-soluble carrier of electrons (45, 107, 182, 187, 215, 221).

As mentioned before, the use of the cofactor NADPH is favored over NADH in reduction reactions in biological sys-

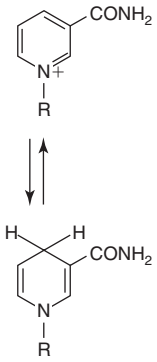

FIG. 4. Structure of the oxidized (*upper*) and reduced (*lower*) forms of the nicotinamide moiety of nicotinamide adenine dinucleotide (phosphate). R indicates the remainder of the coenzyme molecule.

tems; however the high cost of NADPH over NADH has motivated protein engineers to pursue the change of specificity of reductases for NADH in the development of biocatalysts. In this sense, several strategies have been applied, from structure-based protein design (90, 101, 180), consensus based (75), and CASTing (126).

E. Metallic redox cofactors

The most biologically relevant metals are first-row transition metals, notably iron, nickel, copper, zinc, vanadium, and manganese. Occasionally, transition metals combine with second-row transition elements such as molybdenum to form heterogeneous clusters (91, 97). In cells, iron is the most abundant metal and is normally found in the +2 or +3 oxidations states. However, higher oxidation states may be generated transiently in the course of the catalytic cycle of enzymatic reactions (23). Besides single iron ions, proteins can bind a range of iron-containing cofactors, such as heme, iron–sulfur, iron–nickel, or iron–molybdenum clusters (166). The second most abundant transition metal in living organisms is copper. Although less versatile than iron (copper ions present only two redox states compared with six of iron), copper holds a unique position because it presents a fairly high reduction potential (4, 172). Both iron and copper have been recruited for electron transfer, binding and activation of oxygen, and oxidation-reduction of substrates.

Metals resemble protons in that they are electrophiles able to accept an electron pair to form a chemical bond. In this aspect, metals may act as general acids to react with anionic and neutral ligands. The larger size of the metal atom relative to protons is compensated for by their ability to react with more than one ligand, typically two, four, or six ligands. If a metal is bound with two ligands, it will form a linear complex. If the metal reacts with four ligands, the metal will be set in the center of a square that is planar or it will form a tetrahedral structure, and when bound with six ligands, the metal sits in the center of an octahedron (178).

Approximately one-third of the known proteins are metalloproteins, conformed by a scaffold complemented with metal atoms in individual arrays or combined with other metals or with inorganic molecules (97). Inorganic ligands to these metals involve sulfide, water, carbon monoxide, and cyanide. Organic molecules such as homocitrate and dithiols can also so be involved. The most common metal ligands in proteins are cysteine for iron and histidine for copper, although any protein group capable of forming a coordination bond has been found to do so (58).The protein environment may affect the redox potential of the transition metal-based redox centers in a controlled manner, expanding their biological functionality.

Metallocluster-containing enzymes are an important group among metalloproteins composed mainly of iron, nickel, copper, manganese, and molybdenum (66, 97, 185). The reactions catalyzed by them typically involve small molecules such as molecular nitrogen, carbon dioxide, and hydrogen. The most abundant metalloclusters appear to be the iron–sulfur clusters, presenting two, three, or four iron atoms coordinated to protein residues and bridged by inorganic sulfide, with cubane (4Fe–4S) being the prototypical and most ubiquitous (23, 173). The wide variation observed among measured redox potentials of the same cubane configuration has been attributed to a variety of factors, including electrostatic interactions between the metal center and point charges and dipoles in the protein, hydrogen bonding between the metal center and the protein, and the hydrophobicity and solvent accessibility of the metal center. Accordingly, a great effort has been imprinted into the design of metallocluster binding sites by the site-directed mutagenesis of ligands and of other parts of the proteins.

F. Prediction of metal-binding sites in protein sequences

One of the main limitations for the engineering of metalloproteins is the limited amount of information available from a very small number of cases with sufficiently characterized metal binding sites. In contrast to the mainstream in protein research, sequence identity-based search engines such as BLAST (even last generation upgrades) do not allow a robust prediction of whether a given sequence might or might not bind a metal atom (2, 3, 117, 190). This problem was previously addressed by the Metalloprotein Program Project team of the Scripps Research Institute with the construction of the PROMISE database and the Metalloprotein Database and Browser (MDB) (36, 57). These databases collected structural and functional information on metalloproteins, with emphasis on the properties of the metal sites. Unfortunately, PROMISE was discontinued in 2002 and MDB in 2004. Nevertheless, these databases still provide the largest amount of public information available on metal-binding sites.

Metal-binding capabilities are encoded in the primary sequence of proteins and are displayed through the three-dimensional arrangement of specific amino acid side chains (24, 35). Some primary sequence "signatures" for binding special metal cofactors have been identified over the years (*i.e.*, the nature and spacing of amino acids that determines the ligand field for a specific metal site). The predictive capacity of these signatures strongly depends on the length and composition of the intervening sequences between ligand residues, being more informative when short and with low complexity (21, 64, 76, 77, 87).

The number of metalloproteins of known three-dimensional structure is growing exponentially, providing structural biologists with enough information in the search for rules regarding the type and frequency of amino acid residues that participate in metal coordination. It is widely accepted that metalloproteins may constitute as much as one-third of all known proteins. The most common side chain group for iron, copper, and nickel atoms coordination is His, followed by Cys, Asp, and Glu; for zinc and cadmium the

side chains of His and Cys are equally frequent, followed by those of Glu and Asp (58). The coordination number of the metal ions differs significantly among the metal types. However, for the majority of metals, the most frequent coordination numbers are four and six. The preferred coordination number for copper, zinc, and cadmium is four, while cobalt and iron prefers number six (58).

In order to investigate if there is any structural feature common to all metalloproteins, we performed an annotation-based search of the Protein Data Base (PDB) aimed at evidence of transition metal-binding. By the end of 2007, almost 15% of the deposited structures (7001/47403) contained at least one of the following metal atoms: iron, copper, zinc, vanadium, molybdenum, or nickel. The most frequent metal was zinc (4314/7001), which is consistent with previous observations and may reflect a bias in the PDB towards eukaryotic proteins (5, 36, 57). The abundance of the other metal species was as follows: iron (1282), copper (765), nickel (462), molybdenum (159), and vanadium (19).

With the exception of zinc-containing entries, where it was not possible to discriminate whether the metal satisfied a structural or a catalytic role, we tried to elucidate potential correlations between the metal species bound and the folding pattern of the protein. To achieve this, each one of the 2687 PDB entries was classified according to the CATH categorization scheme (Fig. 5) (153). The most frequent class was Alpha Beta (47%), followed by Mainly Alpha (29.9%), Mainly Beta (19.9%), and Few Secondary Structures (3.1%). The most populated architectures were Orthogonal Bundle (16.2%), 2-Layer Sandwich (14.8%), 3-Layer ($\alpha\beta\alpha$) Sandwich, and Up-down Bundle (12.2%), and Alpha Beta Complex (10.8%).

From this analysis, there seems to be a correlation between the folding pattern of a protein and the presence of a metallic center, being significantly more frequent in folds with a high content of alpha helix structures. Possible reasons for this correlation are: (a) the set of amino acid side chains electronically capable of metal coordination (mainly cysteine and histidine, although methionine has also been observed) are less represented in beta sheets (153); (b) that the precise tridimensional arrangement that amino acid side chains required for metal coordination is more difficult to attain in protein surfaces with stronger steric limitations as are frequently found in beta-rich structures; (c) that alpha-rich structures would be more flexible, allowing the conformational adjustment of ligands triggered by the geometry change of the metal atom during oxidation–reduction (47); and (d) the dipole formed by alpha helices may be a natural attractor to charged species, especially in a parallel arrangement of several of these elements of secondary structure, as is the case in helix bundles.

Independently of the reasons for the higher frequency of metal sites in alpha helix-rich folds, there is an important

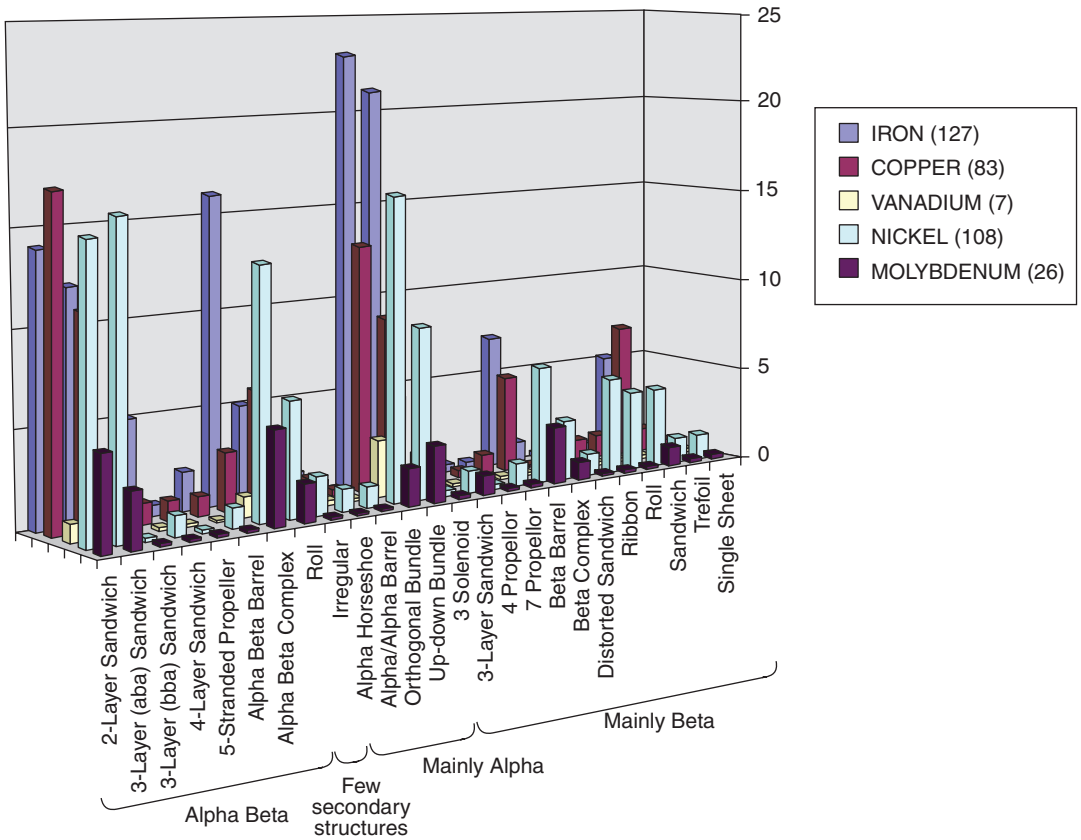

FIG. 5. Distribution of annotated metalloproteins according to their topologies. Individual topologies are organized by their common architecture, and architectures are organized in classes (Alpha Beta, Few Secondary Structures, Mainly Alpha, and Mainly Beta) according to the CATH categorization scheme. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article at www.liebertonline.com/ars).

consequence, since the accumulated dipolar moment of the secondary structures may affect the redox potential of the metal sites. In this regard, it has been demonstrated that the identity of a single amino acid in an alpha helix tunes the redox potential of a ($Fe(SCys)_4$) site in rubredoxin by adjusting the peptide dipolar moment (66, 69).

III. The Protein Engineer's Tool Chest

The undisputed power of genetic engineering is based in its ability to modify the complex properties of proteins by the use of the rather straightforward methods of recombinant DNA. The central dogma of molecular biology states that the biological information flows from the chromosomes (or any other genetic material) to proteins through an intermediate RNA molecule. Nowadays we know that the flux is dynamic and in some instances bidirectional. Nevertheless, the premise remains valid, and any modification performed on a gene will translate into a residue substitution on the encoded protein.

Genetic engineering relies on the isolation of a protein-encoding piece of genomic material, its cloning in an expression vector, and the subsequent manipulation of the sequence to produce altered versions of the encoded protein by two different and equally powerful resources: (a) random mutagenesis followed by selection, also known as directed or molecular evolution, and (b) the rational design of novel properties based on the knowledge derived from the three levels of the protein structure. Each one of them will be described in detail below and significant examples of their application to redox-active enzymes will be discussed.

IV. Directed Evolution

Also called *in vitro* evolution, directed evolution is used in protein engineering to harness the power of Darwinian selection in the evolution of proteins with desirable properties not found in nature (136). There are two main challenges to overcome when using directed evolution, one is the generation of variability and the second is selection. Variability may be incorporated randomly *in vivo*, by the use of "mutator" bacterial strains (deficient in the $3' \rightarrow 5'$ exonuclease activity of PolIII) (199), or *in vitro* by error-prone replication. The advent of the polymerase chain reaction (PCR) allowed the economical and efficient synthesis of practically any sequence independent of the biological source. Whereas template amplification may be achieved by the use of any DNA-dependent DNA polymerase, those from thermophilic sources are easier to handle. Because of their thermal stability, they can be used in several cycles of denaturation of the double-chain DNA, and annealing of a primer and extension of DNA at the optimal temperature of the polymerase, allowing the amplification of large amounts of the target piece of DNA. In this regard, the DNA polymerase I from *Thermus aquaticus*, now produced recombinant in *Escherichia coli*, is probably the most frequently used enzyme for *in vitro* synthesis of DNA. Taq polymerase naturally lacks the $5' \rightarrow 3'$ proofreading activity required for high-fidelity replication, the background rate of misincorporations can be artificially enhanced by increasing the concentration of Mg_2^+, introducing Mn_2^+, by adding inosine or dimethylformamide, or an unbalanced mixture of the four natural nucleotides (33).

Nowadays, it is possible to generate as much variability and with all the characteristics required by the experimenter. The real bottleneck is then the identification of those unique combinations yielding proteins with novel properties, whether it is increased activity, stability to pH, thermal stability, stereoespecificity, new catalytic functions, or others. To relieve this, it is absolutely critical to design a precise detection method preferable by selection, although a well-devised screening scheme might provide significant results as well. Under ideal conditions, a bacterial strain harboring specific auxotrophy markers can be used for complementation. Selection systems are easy to handle but this benefit might be obscured by an intrinsic disadvantage, that cells are potentially able to respond to selective pressures by adapting themselves to novel metabolic conditions that might compensate for the lack of function or even to generate regulatory shortcuts for the overproduction of low-activity variants.

The main advantage of directed evolution techniques is that only the primary sequence of the protein is required, although a good knowledge of the catalytic mechanism allows a better selection design. The drawbacks are that the diversity generation is often limited by technical reasons, and that, even in those cases where the generated diversity is enough to cover the sequence landscape, downstream analysis requires expensive robotic equipment to automate the process. Furthermore, not all desired activities can be easily screened for. Finally, the results of directed evolution experiments are often poorly informative in that the selected mutations schemes are not necessarily valid for other proteins, even if they are closely related (211, 242).

Despite these drawbacks, directed evolution is extremely useful in those cases where there is a clear phenotype to select for. Several redox-active proteins have been submitted to directed evolution protocols for the selection of diverse phenotypes, frequently for industrial and environmental purposes, including oxido-reductase variants with broaden substrate specificity (9, 120) or with altered specificity such as the evolution of vanillil-alcohol oxidase (VAO), a flavoenzyme that catalyzes the oxidation of a wide range of phenolic compounds, to produce vanillin from creosol (220). VAO catalyzes the oxidation of creosol with low efficiency in a two-step reaction. In the first step, creosol is oxidized to vanillil alcohol that is further oxidized in the second step to the alkanone vanillin. The formation of a stable abortive complex between the enzyme-bound flavin and creosol limits the yield of the reaction. Moreover, the second step is inhibited by the competitive binding of creosol (219). After error-prone PCR, seven single mutants capable of producing vanillin from creosol were isolated. The increased activity was apparently due to destabilization of the creosol-flavin adduct formed. None of the substitutions were positioned near the substrate binding site, and therefore, would not have been targeted in a rationally designed strategy.

In another example, the nonheme iron-dependent estradiol catechol dioxygenase was evolved by error-prone PCR for a change in product specificity. Oxidation of catechol substrates is catalyzed by two types of dioxygenases during the degradation of aromatic organic compounds. Intradiol dioxygenase catalyzes the oxidative cleavage of the C–C bond between the two phenolic hydroxyl groups using a nonheme-iron (III) cofactor. On the other hand, extradiol

dioxygenase catalyzes the oxidative cleavage of the C–C bond adjacent to the hydroxyl groups and uses a nonheme-iron (II) cofactor. Although the products are different, it has been proposed that catalysis of both reactions proceeds through a common hydroperoxide intermediate (30, 191). Schlosrich and coworkers used directed evolution to generate variants of class III extradiol dioxygenase with intradiol oxidative activity towards environmentally important chlorinated catechols. Just as in the aforementioned cases, some of the mutations giving rise to altered specificity are hard to explain in terms of their distance to the substrate binding site. Another important result from the analysis was the identification of a switch between extradiol and intradiol cleavage activities, supporting the hypothesis that both mechanisms occur via a common intermediate.

For the Rieske-type three-component enzyme biphenyl oxygenase, which natural function is the oxidative degradation of biphenyl, shuffled variants composed from the *bphA1* genes from *Pseudomonas pseudoalcaligenes* KF701 and *Burkholderia* sp. strain LB400 were selected for the degradation of chlorinated biphenyls, a highly toxic and recalcitrant pollutant (118). Interestingly, the deduced amino acid sequences of the evolved variants showed only a few amino acid changes compared to the original enzymes.

As documented above, error-prone PCR has proved to be a powerful tool for protein evolution; yet, the analysis of a large number of variants relies on the availability of a suitable detection method. Without any doubt, the direct estimation of product is the most reliable way of assessing the success of an evolved enzyme. However, each particular case requires the development and validation of a method that, in many cases, involves the modification of the product to obtain some measurable property. As a consequence, there is an increase in time and reagents needed, not always applicable to a high-throughput format. For redox-active enzymes, the most frequent alternative methods are based on the estimation of used cofactors, co-substrates, and surrogate substrates (210).

The determination of cofactors or co-substrate consumption has the advantage of simplicity and general applicability, however, the uncoupling problem in monooxygenases might lead to the selection of variants with increased production of peroxide instead of the product of interest. Surrogate substrates, on the other hand, have been successfully used with redox-active enzymes. The search of cytochrome P450 variants for the regio- and enantioselective monooxygenation of medium-chain alkanes and carboxylic acids was achieved through the use of *p*-nitrophenolate derivatives of the substrates (165). This is based on the formation of an unstable hemiacetal that decomposes, releasing *p*-nitrophenol which can be monitored spectrophotometrically. The hydroxylation of alkanes has also been successfully tackled using methyl ethers as surrogate substrates (135, 165). In this case, the decomposition of the unstable hemiacetal produced formaldehyde that was detected as an end point product by its reaction with purpald, yielding a purple compound.

The random mutagenesis strategy is further challenged by the compromise between obtaining the substitutions needed to achieve the target property and the accumulation of deleterious mutations (22, 25). In fact, only about half of the random single-base pair substitutions yield changes at the amino acid level, and from these, only five to six other amino acids can really be explored at a given position. As a consequence, only 25% of all possible single amino acid replacements can be recovered. In order to enhance the exploration coverage and also to confine the structural consequences of the perturbation, sequence variation may be introduced by the use of mutagenic oligonucleotides. This strategy requires some knowledge of the three-dimensional structure of the protein to identify the region to be mutagenized, which usually covers a sphere around the active site, or it can rely on residues identified though a general mutagenic/selection experiment as important to evolve certain property. Even though this strategy is site directed, an evolutionary component is added by introducing diversity at several residues at a time, followed by selection or high-throughput screening for a given functional property. We will briefly describe some successful cases in which this strategy was applied.

For dihydrofolate reductase type II (DHFR), a combinatorial approach was used to randomize residues 66–69 which constitute the active site surface. In this protein, only one monomer of the functional tetramer can be occupied by substrate (DHF) and cofactor (NADPH) at a time. As a result, the combination of subunits carrying different mutations, increase the sequence space for activity exploration (192). The most interesting contribution of their work was that an entirely new active-site constellation can be constructed, as long as the participant residues can be simultaneously varied. These results demonstrate the plasticity of the active site and the roughness of the function-sequence landscape.

In another example, the combinatorial mutagenesis strategy was applied to elucidate the functional role of three key residues in cytochrome c peroxidase (189). A distal arginine, originally thought as a stabilizing factor for the negative charge developed during the heterocyclic cleavage of the peroxide bond, in fact stabilizes Compound I and controls reactivity and specificity by restricting the access to the ferryl oxygen atom. The tryptophan residue at position 51 that was considered to hydrogen-bond the ferryl oxygen atom was randomized. The only viable variant obtained at this position contained a phenylalanine; this substitution favored the proton transfer from the substrates to the ferryl oxygen atom, thus increasing reactivity towards phenolic substrates. The third position, tryptophan 192, which is located in the core of the protein, tolerated mutations to polar and positively charged residues, reflecting the complementarity to the environment around this residue which is transiently charged during the reaction mechanism.

Combinatorial mutagenesis has also been used to increase stability of Mn-peroxidase towards peroxide (138). In a more ambitious scheme, Suemori and Irakura applied a systematic combinatorial strategy to simultaneously buildup activity, specificity, and thermal stability of p-hydroxybenzoate hydroxylase (209). Their mutagenesis was directed only to cysteine and methionine residues to explore all 18 amino acid replacements. They selected single mutants for each improved property and they recombined them iteratively, increasing the number of properties to be screened in each round to end up with multiple mutants improved in all four traits. Although this strategy allowed an effective adaptive walking towards particular objectives, while restricting the variability to be searched, the assumption of additive effects leaves out any potentially synergistic interaction.

Finally, the combinatorial mutagenesis strategy has also been successfully tested to change substrate specificity, as demonstrated by Arnold and coworkers who simultaneously explored 11 positions in the substrate binding channel of P450 Cyt BM3, with the aim to find variants able to produce ethanol from ethane (135). As expected, the resultant active mutants had an increased side chain to fill in the cavity in order to fix the considerably smaller substrate. These mutations were complemented with mutations at the reductase domain to increase the coupling of electron transfer.

A more systematic technique based on combinatorial mutagenesis is the so called CAST (Combinatorial Active-site Saturation Test), implemented and successfully applied to expand substrate specificity of lipases (175) and to change enantioselectivity of epoxy hydrolase (26, 174) and of cyclohexanone monooxygenase (42). In a first round of CAST, sets of two or three amino acids with side chains forming part of the binding pocket are randomized simultaneously with the generation of relatively small libraries easy to screen for activity. The hits of the first rounds are used for the next round where other positions are explored. The different regions considered for mutagenesis can be handled in parallel to generate independent libraries from which to build up the next generations until a decision is made of which branch will be used to continue the evolutionary process. With this strategy, the authors were able to increase the enantioselectivity factor, E, of the epoxy hydrolase from 4 to 115, after screening a total of 20,000 variants. In contrast, the same laboratory obtained an enantioselective factor of only 60 after a series of error-prone PCR and screening an equal number of variants (176).

Although CAST requires the sampling of a large number of libraries, the lower demand on screening and the higher performance pays the effort off. These strategies can be further improved by the use of intelligent mutagenic DNA oligonucleotides. The saturation mutagenesis, using NNG/C codons can be replaced by the synthesis of trinucleotide phosphoramidites representing codons for all 20 amino acids (224), avoiding redundancy due to degeneracy of the code, as well as undesired amino acids and stop codons. Thus, the number of variants to screen is considerably reduced, assuring a complete mutant coverage in the screen process. Several techniques have been developed to increase the yield of the mutagenic DNA primers with simultaneous control of the proportion and quality of the mutagenic codons, such as orthogonal chemistry (82).

Naturally, recombination plays a key role in evolution by enhancing the evolutionary rate of an enzyme for a given trait (72, 73). Artificial recombination methods have been recently developed in order to reduce decision-making to a minimum and to avoid laborious protocols aimed to build mutations on top of the already selected ones. DNA shuffling (200) and STEP (243) are two such methods, in which DNA mixes and matches pieces of successful variants to produce better results by mimicking recombination as it occurs naturally during sexual reproduction (27, 211). In the original approach, DNA fragments encoding the proteins of interest were enzymatically chopped up in pieces and allowed to pair among themselves until the whole gene was reconstructed (Fig. 6).

A comprehensive evolutionary scheme has been followed for the modification of a fungal multicopper enzyme. In a first report, the selection of randomly generated variants of the *Myceliopthora thermophila* laccase were selected for a more efficient expression in yeast (31). Ten generations and the screening of 20,000 clones were necessary to obtain a mutant with ~20-fold increased activity named MtLT2. The deduced protein sequence of MtLT2 presented 13 differences when compared with the parental enzyme. From these, only three are probably related to the enhancement of the catalytic performance, whereas the rest might be involved in protein stability. In a second report, this same mutant was subjected to directed evolution selecting tolerance to high concentrations of two different cosolvents in one screening assay (244). The strategy followed relied on the utilization of gradually increased concentrations of acetonitrile and ethanol and the simultaneous selection of high activity and stability. Randomized variability was generated by *in vitro* as well as *in vivo* methods, taking advantage of the recombination apparatus of *S. cerevisiae*. The best mutant obtained displayed a remarkable tolerance toward cosolvents, retaining in 40% ethanol or in 30% acetonitrile the same activity as the parental MtLT2 displayed in aqueous media.

Since the reconstruction method is based on sequence complementarity among pieces, only variation of the same gene whether obtained by *in vitro* mutagenesis or from different homologs with high degree of sequence identity at the DNA level could be included. The assembled product resulted in a mosaic gene with 1 to 4 crossed-over points, harboring the variability encoded by each one and all the different parental sequences (46, 200). The natural limitation of this technology is the high level of conservation required among the parental sequences (over 60%) and that the recombination sites are constrained to those segments with even higher sequence identity. This limitation is even more restraining when distantly related sequences are to be combined into chimaeras.

The rationale behind the evolution of proteins through chimeragenesis is the introduction of variability that has already been proved to be compatible with the structure and function of a given protein. Recently, a series of alternatives to the basic DNA shuffling method have been developed that reduce the identity treshold required for the parental sequences. In the SHIPREC technique (Sequence Homology-Independent Protein Recombination), the two genes to be recombined are linked by a separator sequence harboring a restriction site. This construction is circularized, truncated randomly, and the fragments corresponding to the size of a single gene are purified, treated with Klenow to produce blunt-end DNA, and self-ligated. Digestion of the sequence at the separator size yields a collection of linear fragments of chimeric genes coding for the N-terminal part from one protein and the C-terminal part from the other, with crossovers distributed over the entire length of the gene (Fig. 7). With this method, the soluble P450 cytochrome from *B. megaterium* and the membrane-associated human P450 were recombined. These genes share only 16% identity, and therefore a conventional gene-shuffling strategy would have not been possible. The resulting library was amplified and cloned for characterization as translational fusions to a folding reporter gene (*e.g.*, chloramphenicol acetyl transferase) (196). According to their folding-assessing screen, ~20% of the library was properly folded, and 80% of these clones retained CO binding ability. After screening 2,000 variants for

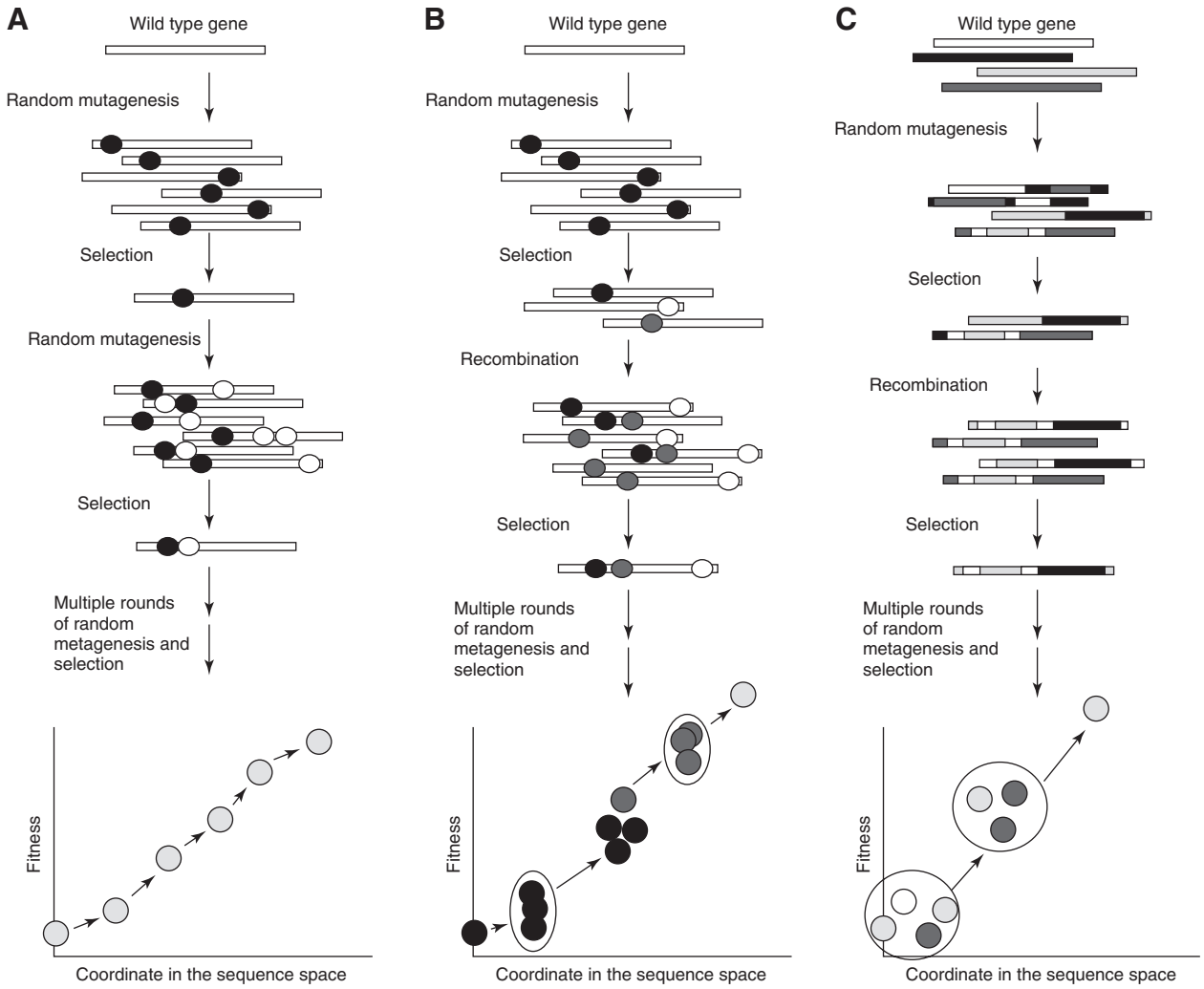

FIG. 6. Performance of different mutagenesis-recombination-selection schemes on the exploration of the fitness landscape of an enzyme. (**A**) The use of random saturation allows the population to travel the sequence space in discrete steps. The strength of the method is that no deleterious mutations are introduced, given the low intensity of the mutagenesis. (**B**) An improvement of the previous method is the recombination of a group of independent mutants on the same gene. The combination of methods will allow the population to bounce through the sequence space faster than the use of each one of the parent methods. Additionally, the positive phenotype of different mutants recombined is expected to add while the negative effect of others is expected to dilute and eventually to loose from the population. (**C**) The most powerful, yet the most difficult, approach is the recombination of different genes, homologous or nonhomologous. In this regard, the population could be expected to use regions of the sequence space that would be forbidden to any one of the parent sequences and also to reach novel fitness peaks, for instance, those that embrace a new catalytic activity on an extant fold (catalytic migration).

deethylation of 7-ethoxyresorufin, activity exhibited by the human P450 cytochrome, the authors were able to isolate two active clones. Both clones had the chimeragenesis site inserted into the first 16 amino acids of the human cytochrome; in one of them these residues were replaced by the sequence of the soluble bacterial enzyme; in the other there was some frame shift that was later recovered, giving an initial different sequence, but in both cases the result was a soluble enzyme present in the cytosolic fraction.

In a third method, named RACHITT (Random Chimeragenesis on Transient Templates), two genes with no identity are recombined. One of the parental sequences is enabled as a transient template as a uracil-containing single strand while the other is chopped up in small pieces with DNAse III. The small pieces are allowed to anneal randomly to their homologous parts of the uracil-containing template and the single-strand intervening fragments are filled with Klenow. Once the extension step is performed, the uracilated template is destroyed. The mosaic products might contain as many as 14 crossed-over intersection points per gene and the recombined pieces might be as small as five base pairs (44). Coco and collaborators used this strategy to construct a library of chimeras from *dszC* genes encoding dibenzothiophene monooxygenase (DBT-MO), which catalyzes the first and limiting step of the *dszABCD* diesel biodesulfurization pathway. They used as parental genes DBT-MO from *Nocardia asteroides* A3H1, which shows a wide substrate spectrum but poor activity and DBT-MO from *Rhodococcus ery-*

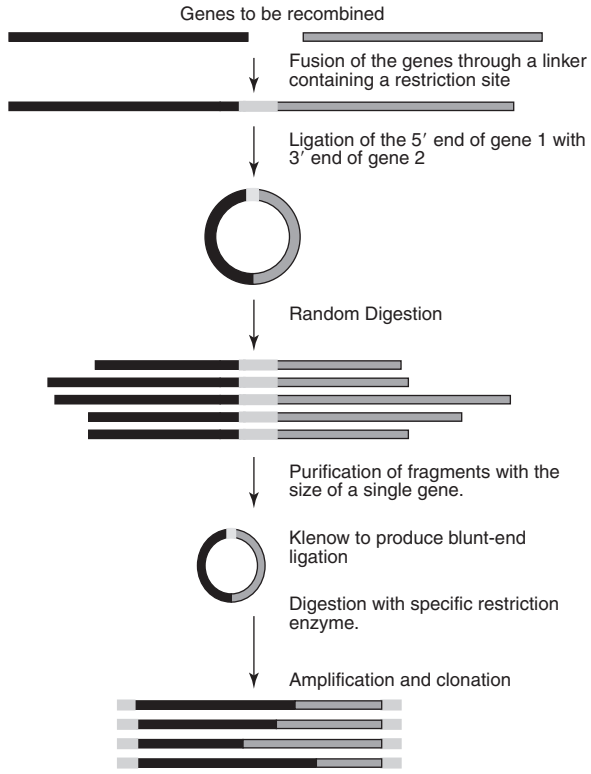

FIG. 7. Experimental strategy to generate chimeras through Sequence Homology-Independent RECombination (SHIPREC). The genes are fused through a linker containing a unique restriction site. The fusion is circularized through a ligase and digested randomly with DNaseI. Fragments of the size of a single gene are purified and treated to produce blunt ends that are ligated back and finally linearized by the specific restriction enzyme. The chimera genes are amplified, cloned into a vector, and screened for function.

thropolis IGTS8, that shows higher conversion rates, but higher specificity for sparsely alkylated DBTs. Screening of 175 chimera proteins allowed identification of variants with high activity and broad specificity, while previous extensive efforts of random mutagenesis on the target genes by the same laboratory failed in improving the properties of these gene products.

In the last method reviewed here, called structure-based CHEMeragenesis algorithm (SCHEMA), an algorithm was developed to determine the number of interactions that would be disrupted upon formation of a hybrid enzyme at every single position when several parents are used to recombine (226). The automatic energetic evaluation of all possible chimeragenic sites allows the identification of independent folding fragments that can be recombined, maintaining the integrity of the three-dimensional structure of the protein, increasing the proportion of folded chimeras. The method has proved to be successful in generating a library with high diversity (an average of 72 amino acid substitutions with respect to the closest parent sequence), and high content of folded chimeras (~50%). The library was formed when eight fragments of cytochrome P450 heme domain genes from three bacterial parents, *B. megaterium* (BM 3) and its *B. subtilis* homologs (A2 and A3), were recombined (154). The resultant library was screened for thermostability (125) and for activity towards a set of substrates (121). A significant number of chimeras were found that were more stable than the parental enzymes. These chimeras also showed a good correlation between the presence of particular fragments and stability, so that a combination of those fragments expected to confer the higher stability behavior was assembled. Regarding activity, a set of 14 chimeric proteins was tried for a set of substrates, finding in all cases that for the entire substrate set, one chimera outperformed even the best parental enzyme.

V. Rational Design

Along with directed evolution, rational design has been exploited as a powerful approach for unveiling the basic principles of redox-active mechanisms. In this section we will present the most effective methods, both genetic and chemical, for the exploration of redox properties.

A. Loss of function approach

From the technical point of view, the simplicity of the site-directed substitution methods allows the production of any possible variation in the sequence of a protein in a fast and accurate manner. It is not the aim of this paper to analyze the techniques for single-site mutagenesis in detail, but only to identify some general strategies applied to the study of redox enzymes.

There are countless reports of the site-directed substitution of redox-related residues, mainly cysteine, methionine, and histidine, in order to characterize the effect of side-chain modification on the molecular mechanism. As a consequence of this systematic approach, a series of motives or signatures has become available [*e.g.*, the CxxC sequence, employed by thioredoxins and glutaredoxins as a redox-sensing site (77) or the CxxS sequence involved in methionine sulfoxide reduction (76,119)]. In the case of cofactor binding sequences, the best known are the nucleotide binding sites, where despite minor divergence, a core assembly of secondary structures with conserved sequence can be easily recognized using standard bioinformatics tools (181, 235). The robust prediction of metal binding is relatively more difficult, since the ligand atoms are usually spread apart in the primary structure of the protein without conservation of the intervening sequences. Despite the difficulties, highly represented sites such as the zinc-binding motif allowed the composition of a sequence pattern with significant prediction capabilities (5, 64, 158).

B. Incorporation of non-natural amino acid residues

The availability of chemical functions afforded by natural amino acid residues is limited to that of acids, amides, alcohols, basic amines, and thiols. The possibility of expanding the natural repertoire of reactive groups in proteins has been actively pursued by protein engineers. Amino acid analogs have been used to unveil new roles of individual residues in protein function, structure, folding, and localization. For instance, it is now possible to incorporate fluorescent labels for protein-trafficking studies, to add photoactivatable crosslinkers for protein–protein interaction studies, or to make post-translational modifications to exogenously produced proteins (68, 128) .

Novel amino acids can be introduced into recombinant proteins in either a residue-specific or in a site-specific fashion. These methods are complementary: residue-specific incorporation allows engineering of the overall physical and chemical behavior of proteins and protein-like macromolecules, whereas site-specific methods allow mechanistic questions to be probed in atomic detail. The most common strategy for incorporating amino acid analogs into proteins relies on reading through amber stop codons (UAG) in mRNAs by a suppressor tRNA that is aminoacylated with the desired non-natural amino acid (148).

The incorporation may be achieved *in vivo* or *in vitro*. The basis for *in vitro* systems is the use of a suppressor tRNA that is chemically aminoacylated, whereas *in vivo* systems rely on the misacylation activity of a mutant aminoacyl-tRNA synthetase. The essential requirement for the *in vivo* method is that two of the recognition elements (*i.e.*, the suppressor tRNA and the aminoacyl-tRNA synthetase) behave orthogonally, that means that there they should present a strong specificity between them, avoiding cross-interaction with the rest of their cellular analogs (10, 130, 186). Original efforts to develop a stringent orthogonal system in *E. coli* were marginally successful until a proficient selection system was developed by Schultz and coworkers (230). They submitted a library of tRNA mutants (derived from a heterologous suppressor tRNA) to negative and positive selection rounds in the absence and presence of the cognate synthetase. In the negative selection stage, the tRNA library is introduced into *E. coli* along with a mutant barnase gene in which amber nonsense codons are introduced at sites permissive to substitution by other amino acids.

When a member of the suppressor tRNA library is aminoacylated by an endogenous *E. coli* synthetase (*i.e.*, that it is not orthogonal to the *E. coli* synthetases), the amber codons are suppressed and ribonuclease and barnase are produced, resulting in cell death. Only cells harboring orthogonal or nonfunctional tRNAs can survive. All tRNAs from surviving clones are then subjected to a positive selection round in the presence of the cognate heterologous synthetase and a β-lactamase gene with an amber codon at a permissive site. tRNAs that can function in translation and are good substrates for the cognate heterologous synthetase are selected on the basis of their ability to suppress the amber codon and produce active β-lactamase. Therefore, only tRNAs that (a) are not substrates for endogenous *E. coli* synthetases, (b) can be aminoacylated by the synthetase of interest, and (c) function in translation, will survive both selections (230).

Nevertheless, the use of amber codons presents an intrinsic limitation: the natural propensity of the ribosome to recruit release factors as soon as an amber codon reaches the A-site. The suppressor t-RNA must then compete for the release action with the concomitant reduction of the overall efficiency to $<$50%. Higher suppression efficiency may be achieved by the use of one of several strategies: the design of non-natural codon/anticodon base pairs; selective expansion of codons from three to four or five nucleotides; the induced accumulation of the suppressor tRNA, or the selective inactivation of temperature sensitive release factors (231). Despite the relative success of all these alternatives, efficiencies are still below optimal values. Moreover, as the number of amber codons in a gene increases, the efficiency of non-natural amino acid incorporation decreases.

An upgraded version of the orthogonal method has recently been reported that allows the evasion of its natural limitations by adding a specialized ribosomal particle, named "ribo-X" to the *E. coli* machinery able to specifically translate the UAG-containing orthogonal mRNA, but that also appears to present reduced affinity for the cellular release factor RF-1 (229). The design of ribo-X bases on the recognition of the Shine–Dalgarno sequence in the mRNA by the anti-Shine–Dalgarno sequence in the ribosome. Site-directed substitution of the Shine–Dalgarno sequence in an encoding sequence along with the complementary substitution in a copy of the 16S rRNA generates an orthogonal pair specific for the translation of mutant mRNAs (169, 170). Ribo-X, not being part of the cellular machinery, is tolerant to mutations that would otherwise cause lethal effects. With an experimental strategy that combined rational design and directed evolution, Chin and coworkers evolved orthogonal ribosomes that are more efficient in the translation of amber codons through the manipulation of the A-site encoding fragment of 16S rRNA. The authors demonstrate that the mutant ribosome enhances the suppression efficiency of single amber codons threefold, compared to the wild-type ribosome, and 22-fold for two amber codons in the same gene. Even further, ribo-X does not augment read-through of stop codons other than amber. The development of ribo-X adds a powerful tool to the protein engineer's tool chest and will undoubtedly facilitate protein engineering for a wide array of application.

C. Chemical synthesis of proteins

Biological protein synthesis, even enhanced by the most powerful adaptations, limits the components of proteins to α-amino acids. In contrast, chemical synthesis allows the facile introduction of non-natural structures into proteins (111, 112). The basic chemistry of peptide synthesis comes from the beginning of the 20th century after the work of the Nobel Prize winner Emil Fischer. Early liquid-phase methods were substituted in the late 1960s by more efficient and controllable solid-phase alternatives that were also easily automated. However, the cumulative loss of product in current solid-phase methods still limits the length of routine preparation of peptide fragments to 50–60 residues.

Recently, the same principles of polymer-supported peptide synthesis have been applied to develop the native chemical ligation method (Fig. 8) (55, 56). Substrate peptides can be protected or unprotected, and coupling can occur in an aqueous or organic solvent, in solution, or on a solid support. After this method became established, a significant number of complex proteins were produced by convergent synthesis, including integral membrane ion channels and glycosylated proteins (12, 39, 63, 106, 114).

The field of chemical synthesis of proteins is vast and the different methods for the generation of peptide backbones and for the chemical ligation reactions have been recently reviewed (56, 88, 89, 115, 147). Thus, we will focus on the impact of this technology on the development of redox-active protein analogs that could not have been obtained by biological means.

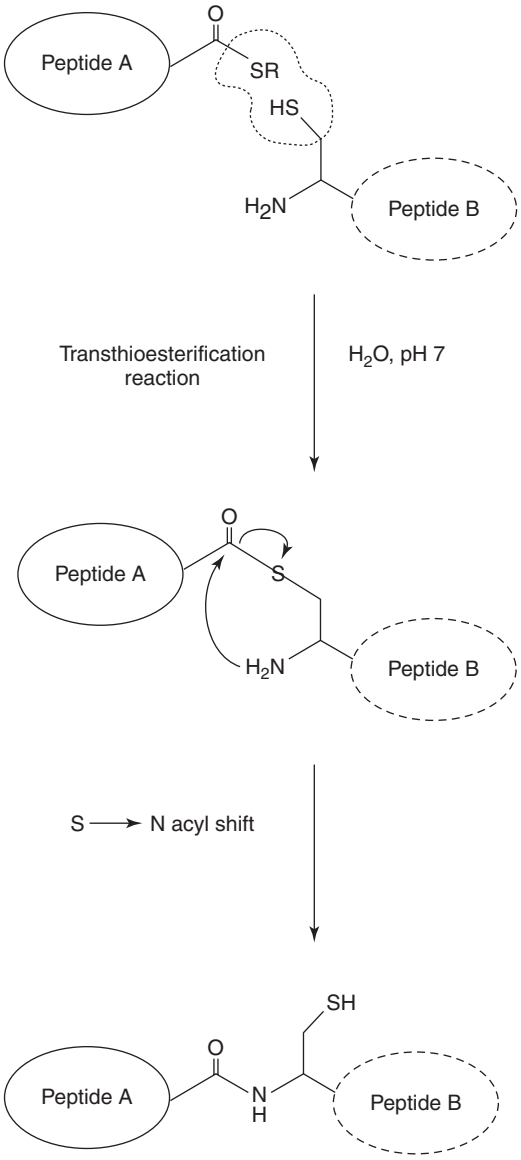

FIG. 8. Basic chemistry of the Native Chemical Ligation method. A peptide containing a C-terminal thioester reacts with another peptide containing an N-terminal cysteine, in the presence of an exogenous thiol catalyst. The product rearranges irreversibly under the usual reaction conditions to form the desired amide bond. A limitation of this technique is that cysteine has to be part of the produced protein at a suitable place.

D. Protein engineering by semisynthesis

Currently, most protein analogs are generated by genetic and chemical methods. Nevertheless, difficult cases involving more drastic modification or complex experiments that require site-specific markers and labels may get solved by an intermediate approach called semisynthesis, a crossroad for the complementary fields of physical biochemistry, chemical, and molecular biology. Semisynthesis is based on the chemical manipulation of the protein itself, allowing the preparation of homogeneous defined analogs using a limited number of chemical steps on synthetic intermediates derived from the native protein (62, 150, 151). The theoretical framework was established by Offord and Rose after exploring the use of hydrazone- and oxime-forming reactions for chemically ligating synthetic and recombinant peptide fragments (80, 81, 179). The field received a recent impulse by the discovery of protein splicing, a post-translational process that proceeds through a branched protein intermediate releasing an internal protein sequence, termed an intein, from a protein precursor (70, 239). During the splicing process, the intein self-catalyzes its excision and the ligation of the flanking protein regions, termed exteins (Fig. 9) (149). To date, >400 intein sequences from bacteria, archaea, and fungi have been documented in the Intein Database (164). Further development of this natural phenomenon resulted in the expressed protein ligation method (141, 143, 239).

The objective of most semisyntheses is the creation of a mutation at a single site in a protein (142, 227). The information defining the target may include the deduced sequence of the protein, as well as a three-dimensional model of its structure based on crystallographic or NMR data, or even a homology-based reconstruction. Each of the phases presented might involve several chemical and physical processes and the potential for variation is vast. An alternative scheme would be stepwise semisynthesis, where subsequent chemistry is performed with the whole protein. This technology has enabled the preparation of semisynthetic proteins aimed towards the investigation of numerous biological processes and biochemical mechanisms including nonredox metalloproteins (14, 79), nonmetallic redox enzymes (65, 98, 177), redox metalloenzymes (228), and for the incorporation of spin-labeled amino acids (13).

A particularly interesting case of semisynthesis applied to the study of redox-active intermediates is the characterization of the intermolecular radical propagation pathway in ribonucleotide reductase. Class I ribonucleotide reductase (RNR) catalyzes the reduction of nucleoside diphosphates to deoxynucleoside diphosphates. The enzyme from *Escherichia coli* is composed of two homodimeric subunits (R1 and R2). Whereas R1 binds the nucleoside diphosphate and the allosteric regulatory nucleotides, R2 harbors a diferric-tyrosyl radical (Y122·) cofactor that is essential for nucleotide reduction. Numerous studies using isotope effects, mechanism-based inhibitors, site-directed mutants, and electron paramagnetic resonance, suggested that the reductive activity of the enzyme requires the propagation of the Y122· radical from R2 to the active site C439 residue in R1 (193, 205). The unusually distant position between both sites (>35 Å) entailed the existence of at least one intermediate position for the productive transfer of the radical, possibly other tyrosine residues, as was demonstrated by site-directed mutagenesis (15, 67, 183). However, the substitution of tyrosine residues by phenylalanine not only perturbed the reduction potential of the side chain but also disrupted the coupling of proton and electron transfers. Since the protonation state of a residue modulates its reduction potential, the absence of a deprotonable group in phenylalanine made the interpretation of the mutant phenotype difficult. The generation and transport of protein-based radicals occurs by proton-coupled electron transfer, a quantum mechanical tunnel effect that results in essential constraint, since proton transfer

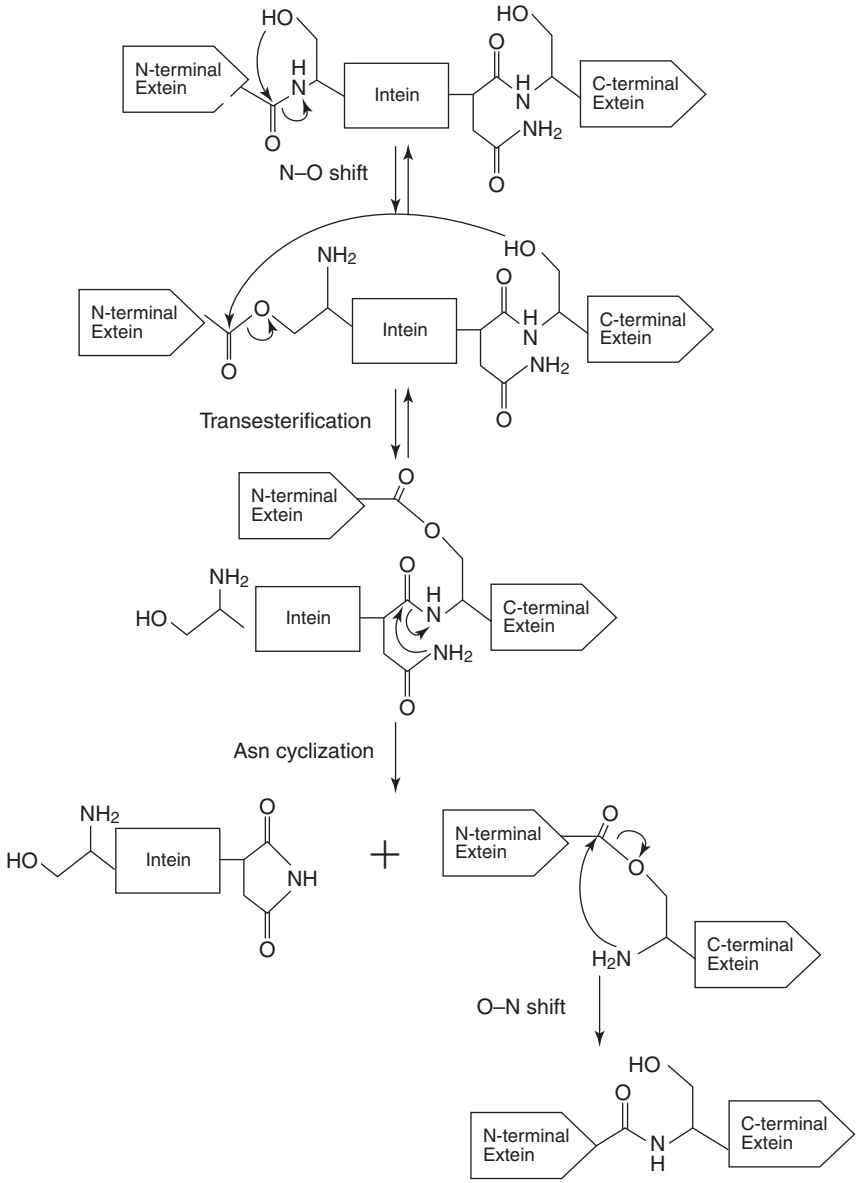

FIG. 9. General mechanism for intein self-catalyzed cleavage. The N-terminal splice junction is activated by a N–O or N–S acyl rearrangement at the intein N-terminus that moves the N-extein to the side chain of the Ser/Cys at the intein N-terminus, forming the linear ester/thioester intermediate.

is limited to short distances relative to electron transfer (171, 202).

In order to overcome these limitations, the authors synthesized semisynthetic versions of R2 harboring the site-directed substitution of the putative intermediate position Y356 by NO_2Y, F_nY, or aniline side chains (37, 194, 195, 240). The rationale behind these substitutions was that analogs might present altered nucleotide reduction relative to their different ability to be oxidized compared to tyrosine. Analysis of the pH rate profile of these analogs suggested that the mechanism of long-distance intersubunit radical/proton transfer in RNR proceeds with electron transfer prior to proton transfer. Radical transfer through position 356 only becomes rate-limiting upon raising the reduction potential of the residue at that location and is not affected by the protonation state of either the ground state or oxidized amino acid.

Thus, semisynthesis is a modern and broadly applicable technique that permits the site-directed modification of complex protein targets and may be exploited as a powerful tool to characterize redox-active proteins (163, 203, 204).

E. De novo design of redox-active enzymes

This is perhaps the most challenging approach for the design of redox-active enzymes. It requires both a knowledge of protein folding and stability, and of coordination chemistry (1, 241). In order to achieve this goal, two alternative approaches have been envisioned. The first one relies on the initial design of a stable scaffold and the subsequent rational incorporation of redox-reactive/related residues. In this regard there are four examples that deserve mention. The strategy followed by Hellinga and coworkers uses the structure of thioredoxin, a small protein that does not contain metal centers, as template to construct the framework for a metal binding site and an oxygen binding pocket. They used the automated program DEZYMER (96) to identify the possible positions to introduce three histidine residues to form the binding site for a single metal center (18). From 200 possible sequences, the authors selected six that placed the metal center in three different environments: on the surface, in a groove, or deep inside the protein. All three constructed vari-

ants were able to bind not only iron, but all first-row transition metals. In a second stage, the authors monitored three different activities commonly catalyzed by metals in proteins: the dismutation of superoxide, the reductive cleavage of hydrogen peroxide (Fenton chemistry), and of molecular oxygen (Undenfriend chemistry) to produce the corresponding free radicals. The constructed enzymes were able to catalyze all three reactions to different degrees with a location-dependent effect of hydroxyl binding on pKa as well as on the redox potential of the metal center, suggesting that location of the metal active center can be used to modulate catalytic activity and specificity.

In the strategy followed by DeGrado and collaborators, they departed from an heterotetrameric helical bundle fold able to conform a diiron site (17, 110, 116). The sequence of the protein was obtained by an automated method (43, 159), a binding pocket was subsequently chiseled in the core of the bundle, the affinity towards the substrate estimated by direct means and finally, the ability of the construction to perform the catalytic oxidation of a model substrate was demonstrated (96). The obtained activity was low but significant and presented saturation kinetics.

The strategy of Tommos and collaborators for the construction of a *de novo* designed quinone protein departed from a stable three-helix bundle (108). This scaffold was able to incorporate redox-reactive residues such as tryptophan to explore the structural basis of some free radical enzymatic reactions (105). Using the same scaffold, Hay and collaborators introduced designed modifications of the bundle core that allowed the covalent binding of 2,6-dimethylbenzoquinone. The bound quinone was able to perform redox cycling with electrochemical parameters close to those expected for a two-electron redox process at room temperature (48).

In the last case, Haehnel and coworkers based their *de novo* design of redox-active enzymes on the chemical synthesis of immobilized four-alpha-helix bundles (93). They synthesized a library of 352 heme proteins with a single ligating histidine residue as the proximal iron ligand and enough space for the binding of a sixth iron ligand, such as water or oxygen. Two of the variants were not only able to bind iron, but also to perform the oxidative opening of the porphyrin moiety yielding biliverdin, by following the same intermediate steps catalyzed by heme oxygenases.

The alternative approach is that of Hecht and collaborators who use a combinatorial strategy for the generation of variability, with emphasis on the explicit location of polar and nonpolar residues but not on their identity (146). In doing so, variability can be generated without compromising the protein core stability. Because the precise identity of each polar or nonpolar residue is not specified, this method allows the construction of large libraries with enormous combinatorial diversity. Among other proteins, the group has generated a series of novel heme-binding variants able to perform a number of functions of natural heme proteins (94). The redox potential of these proteins ranged from -112 to -176 mV, close to the midpoint reduction potential of unbound heme (-220 mV).

VI. Emerging Fields

Here we will present some fields that may boost the investigation of redox phenomena by the application of some of the protein engineering approaches mentioned in the previous sections.

A. Redox-active ribozymes

One of the advantages of protein engineering methods is that its basic theory as well as many of its experimental tools can be expanded for the study and manipulation of RNAs and DNAs. It has been documented how a combination of site-directed mutagenesis and directed evolution techniques were applied for the selection of ribosomal particles with novel functions for the design of orthogonal translation systems (229). Since the discovery in the early 1980s that catalytic RNA molecules are naturally endowed with endonuclease and self-splicing activities, rare RNA-based molecules with novel activities have been isolated in the past yielding binding (aptamer) or catalytic (ribozyme) species (28, 236).

Redox reactions are a key part of all living systems, but so far, only proteins are naturally able to perform them. In a pre-protein world (the hypothesized RNA world), the burden of catalyzing redox reactions might have been borne by RNA molecules (16, 234). An essential observation is that many redox-sensitive cofactors (*i.e.*, NAD, FAD, or cyanocobolamin) harbor a ribonucleotide moiety and may represent a vestige of ancient RNA-based cofactors.

Synthetic RNA templates are able to evolve *in vitro* as aptamers for redox cofactors such as hemin, FMN, riboflavin, FAD, and cyanocobolamin (32, 123, 131, 184, 212). The ability of an aptamer to discriminate between different nucleotides was demonstrated by directed evolution experiments in which an aptamer against FMN or FAD was evolved to bind GMP by partially randomizing the parental RNA sequence and selecting for binding to the new ligand (95). The new anti-GMP aptamers could no longer bind FMN or FAD, even though no negative selections against FMN or FAD were carried out. While the sequence differences between the parental and the new aptamers ranged from 20% to 57%, as few as three nucleotide substitutions were found to alter specificity from FAD to GMP, indicating the existence of fine-tuning specificity determinants, as in proteins.

Ribozymes can covalently self-incorporate NAD and CoA by the same mechanism that is normally used for the insertion of guanosine into the RNA during the initiation of Group-I splicing (29) or by developing new catalytic activities through *in vitro* directed evolution. In the last example, a pool of synthetic RNA was incubated with phosphate in a column, and variants that could append themselves to the column were selected. These variants were able not only to incorporate phosphate but to use any molecule containing a phosphate moiety as substrate. Subsequent rounds of site-directed mutagenesis and selection on the original ribozyme sequence resulted in a remarkable species able to synthesize RNA-linked NAD, FAD, and CoA from their precursors and the 5′-terminal ATP of the ribozyme. (99, 100)

So far, we have documented evidence that RNA molecules might not only interact with nucleotide cofactors as aptamers but also that it is possible to evolve ribozymes able to bind them covalently. Despite this significant advancement, the long-standing question whether RNA molecules would be able to perform redox reactions remained unanswered (104). Soluble cofactors such as NAD or FMN are able to oxidize or reduce substances, albeit at a extremely low rate (156).

Protein scaffolds are required for rate enhancement, although there is not a stringent requirement for the cofactor to be covalently bound, as has been observed for many of NAD-dependent enzymes. In consequence, once an RNA molecule was able to tightly bind the cofactor, the investigation of whether this aptamer could actually be bred to enhance the nucleotide catalytic rate was an obvious question.

The first demonstration that it is possible to select RNA sequences able to perform the oxidation of a substrate with a significant rate enhancement was recently achieved (213). In this report, Suga and coworkers developed an elaborate *in vitro* directed evolution strategy for the selection of ribozyme variants able to efficiently oxidize ethanol using NAD^+ and Zn^{2+} as cofactors. In this selection scheme, a pool of randomly synthesized RNA sequences primed with guanosine-5′-monophosphorothioate (GMPS); the thiolate group was then used for the incorporation of a benzyl alcohol derivative in order to generate a substrate–RNA conjugate. The conjugated pool was incubated with biotin hydrazide in the presence of NAD^+. If the conjugated alcohol group was oxidized by NAD^+, the resultant aldehyde could be coupled to biotin and the self-tagged catalysts separated by streptavidin capture. Two selections were carried out in parallel, one with 100 m*M* Mg^{2+} as sole divalent cation and the other with a mixture of Mg^{2+} and 0.5 m*M* Zn^{2+}. Interestingly, catalysts were only obtained in the presence of Zn^{2+} after 15 rounds of *in vitro* evolution. The stringent requirement for Zn^{2+} and NAD^+ is consistent with the mechanism of natural alcohol dehydrogenases. Based on the estimated background rate, the best performing ribozyme, Ribox2, achieved a single-turnover rate enhancement of $>10^7$-fold. Subsequently, the same ribozyme was shown to catalyze the reverse reaction, the reduction of benzaldehyde by NADH (214). Furthermore, the reaction progressed still in the presence of only NADH and FAD, indicating the operation of a multicomponent redox relay for the regeneration of NAD^+. Subsequent evolution of Ribox2 produced second generation variants able to catalyze the reaction ~25% more efficiently, at the same time being more robust (160).

Taken together, these demonstrations shed some light on our understanding of the evolution of metabolism in the RNA world. The use of NAD and FAD as cellular redox coins is ubiquitous in modern metabolism, and presumably would have been equally widespread in the RNA world. The relatively easy evolution of nucleic acid catalysts that utilize such cofactors implies that the early paths to the establishment of these nucleotide-based structures in biology would have been common.

Structural and mechanistic properties of ribozymes may be translated into the more stable DNA molecule in the form of deoxyribozymes. Although there is no known example of a deoxyribozyme in nature, catalytic variants selected *in vitro* to perform peroxidations were observed for hemin aptamers (212). Alternatively, the translation of the catalytic function encoded by a ribozyme with RNA ligase activity into a deoxyribozyme can be obtained by directed *in vitro* evolution of the translated sequence (161). In this report, Joyce and coworkers demonstrate that the basic chemistry of the ribozyme catalytic activity could be detected in the evolved deoxyribozyme although the value of the catalytic parameters and the stereospecificity were not preserved. Interestingly, the two endpoints of the evolutionary transition are mutually exclusive: when either the ribozyme is prepared as the corresponding DNA or the deoxyribozyme is prepared as the corresponding RNA, there was no detectable activity. An equivalent treatment of redox-active ribozymes might produce a light but robust redox-deoxyribozyme.

The design of functional redox-active polynucleotide scaffolds might fulfill unexpected niches in redox biochemistry. Not only for the resolution of fundamental questions on the origins of life, but also as therapeutic agents. Catalytic sequences might be transfected as parts of larger fragments susceptible to self-processing. Once activated, high affinity aptamers for the cofactor would be expected to self-load (even covalently) with the necessary conformational adjustments for cofactor stimulation. In this form, redox-active ribozymes would be enabled for the intracellular execution of defined redox reactions or simply for fine tuning of the cellular redox status. Even further, the conventional phosphodiester bonds of the backbone may be substituted by peptide bonds in the form of PNA (peptide nucleic acids) able to form extremely stable complexes with great resistance to both nuclease and protease (129, 144, 233).

B. Manipulation and design of intramolecular electron transfer pathways

Electron transfer in proteins is one of the few biological processes based on quantum mechanical tunneling. The kinetics of intraprotein electron transfer becomes temperature-independent at temperatures <100°K, which is an undisputable feature of quantum tunneling reactions. While thermal energy may be insufficient to classically carry the electron over the insulating barrier imposed by the protein medium, quantum tunneling through the energy barrier is still possible allowing electron transfer in biological systems to proceed rapidly over large (10–15Å) distances.

Long-range intraprotein electron transfer is fundamental to respiration, photosynthesis, and other redox metabolic reactions. Participant redox-active proteins have been selected by their ability to transfer electrons between donors and acceptors in a controlled, efficient, and specific manner. Given its preeminent role in biochemistry, the topic of biological electron transfer has been systematically studied for >50 years. At the beginning it was studied from a merely chemical point of view, but more recently it has been the subject of numerous investigations by biochemists and molecular biologists. In particular, after the seminal characterization of the electron transfer processes at photosynthetic reaction centers (139), the search and characterization of electronic pathways inside proteins has been intensely pursued.

Nowadays, the accumulated knowledge of the basic principles of electron transfer theory, supplemented by a vast amount of experimental data obtained from natural systems, allows the enunciation of the rules followed by natural engineering of electron transfer proteins. In general, we are interested in unveiling what elements of their design are important for function and which are not; in particular, we consider it is fundamental to learn how the intervening organic medium components affects the electron transfer pathways. This issue has important consequences for the design and optimization of redox-active proteins.

Theoretical calculation of the reaction rates in systems involving thousands of atoms at a detailed molecular level pre-

sents an enormous challenge, even when the structure of these systems is known. The protein environment is composed of a relatively compact arrangement of amino acid side chain atoms connected by the covalent skeleton of the backbone. Additionally, numerous low-energy contacts occur inside the protein such as hydrogen bonding, electrostatic and Van der Waals interactions. Donor and acceptor redox centers are usually scattered throughout this matrix. The transfer efficiency depends on the ability of the electron wave function to penetrate the classical forbidden insulating barrier between the donor and acceptor. The higher the barrier the more dramatically the electron transfer rate decays with distance. The highest value for the barrier would be found in vacuum. The presence of an intervening organic solvent where the positively charged nuclei can interact favorably with the electron reduces the barrier. A typical protein medium presents a barrier between these extremes (139). No matter the value for the barrier, the rate of electron tunneling will be fastest over short distances (132).

There are two main models that consider which part of the protein environment is more significant in the electron tunneling between a redox pair. The first one, originally suggested by Beratan *et al*. is that specific electron tunneling pathways exist in which covalent bonds are predominantly used, hydrogen bonds are ancillary, and through-space routes are rarely used (20). According to this model, the electron tunneling pathways depend on the detail of the three-dimensional structure of the protein. In this model the overall shape of the electron transfer routes are far from straight, branching and assuming a significant breadth, resembling pathways or corridors.

The method based on the tunneling pathway model searches for the shortest set of connected bonds and short through-space gaps to define a path between redox centers, and then applies a rate penalty for each bond and a greater, distance dependent penalty, for each through space gap (19, 152, 198). Applying this method to a series of analogous reactions allowed the parameters to be adjusted to fit existing experimental rates. This method successfully identifies protein regions that are more or less well bonded and are generally correlated with faster or slower tunneling.

In the original theoretical framework envisioned by Marcus (132, 208) and subsequently developed by Gray and Winkler (85), three factors determine electron transfer within proteins: nuclear reorganization energy (λ); the electronic coupling strength (H_{AB}) of involved redox-active sites; and the distances between redox-active sites (R). The model suggests that different protein secondary structures mediate electronic coupling with different efficiencies, a notion supported by experimental evidence. Analyses of electronic coupling strengths in Ru-modified proteins suggest that the efficiency of long-range electron transfer strongly depends on the protein secondary structure: sheets appear to mediate coupling more efficiently than α-helical structures, with hydrogen bonds playing a critical role in both. (85, 86, 122, 237).

The variations in coupling efficiencies among different protein secondary structures could have important functional consequences. In subunit redox enzymes, the structure between subunits may play a key role in directing and regulating electron flow (Fig. 10). An important inference from this model is that the electron tunneling pathway has been optimized by natural selection to guide the electron, so that changes to the medium, for example, through mutagenesis, will perturb the electron transfer rate. The evolutionary implications of this and the second model will be discussed below.

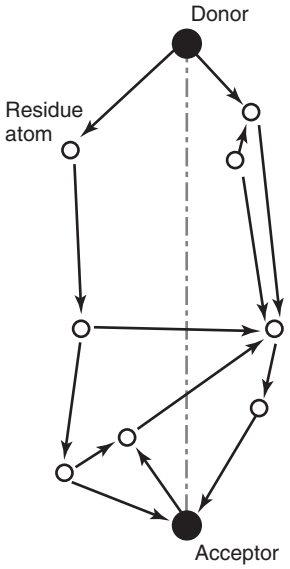

FIG. 10. Electron transfer between redox centers in proteins I. According to the pathway-tunneling model, only a discrete set of atoms located between the donor and acceptor sites will be actively involved in electron transfer forming defined transfer routes. These residues may have been selected for faster or more efficient transfer.

The second model, originally suggested by Dutton and coworkers, assumes that the electron tunneling pathway can be approximately described as a straight line with a width defined by the sizes of the connecting donor and acceptor. In support of this model, they plotted the logarithm of the rate as a function of the distance between donor and acceptor taken from various biological systems, creating a Dutton plot (139). An improvement of the model was performed later with the consideration of the slope as dependent on the packing density of atoms in the protein environment confined by the straight line connecting the redox centers (155). According to the concept of the Dutton plots, the electron transfer rate would be determined only by the distance between the redox pairs, with specific electron tunneling pathways being insignificant in biological electron transfer, which means that the velocity of the electron would remain constant, independent of travel through a covalent or a hydrogen interaction as if the connecting atoms form a straight line. Additionally, the identity of the protein atom in the pathway would be irrelevant, so that changes by mutation would have no impact in the electron rate (Fig. 11).

Experimental support for this model comes from the analysis of structural data of redox proteins, with the observation that the protein packing in the volume between redox centers is indistinguishable from that of other parts of the same protein (presumably not involved in electron transfer) (155). Additionally, a clear tendency was observed for redox centers to be located within 14Å of each other. In the case of long-range tunneling involving more than two centers in different proteins, it was concluded that natural proximity (within 14Å) together with thermal activation, rendered the decay of the electron transfer rate between them linear, in contrast to the exponential decay that occurred in any other direction (155). In this model the protein matrix can be viewed as a homogenous medium lacking detailed structure, whose only purpose is to providing virtual orbitals for the

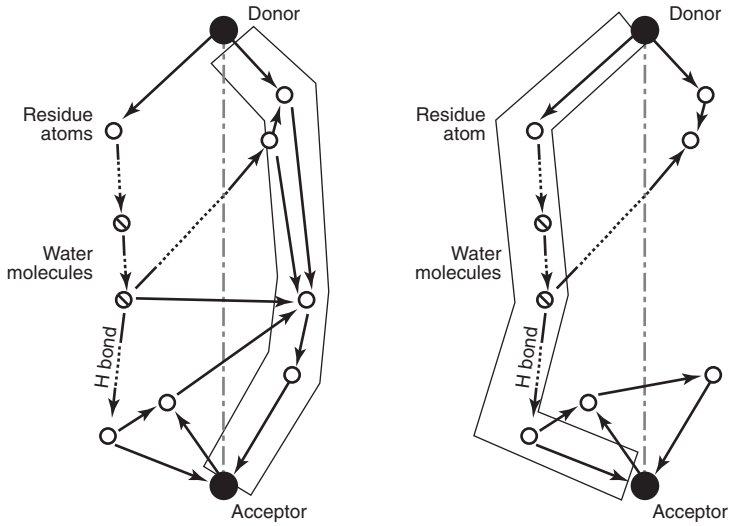

FIG. 11. Electron transfer between redox centers in proteins II. In the absence of defined routes, electrons may travel freely across the protein volume, the shortest path being the fastest. In this case, nonprotein elements, such as bonded water molecules, may also be involved (*left*). Modification or removal of a residue across the preferred route will be automatically substituted by another one, without evident effect on the enzyme performance, as long as the new pathway remains effective (*right*).

electron lowering the tunneling barrier and making long-distance transfer between redox sites possible. Even further, conserved amino acids and structural motives in these regions may have many other biologically important functions with their own selective pressures not directly related to electron tunneling.

Additional considerations enriching the theoretical framework for the modeling of intraprotein electron transfer include the dependence on driving force, the nature of the medium, electrostatic interactions, protein molecular dynamics, the concept of single and multiple electron currents, the effect of water molecules, conformational gating and coupling, and Brownian dynamics (34, 38, 52, 71, 109, 127, 197, 198, 206, 207). In the rest of the section, we would like to focus on the evolutionary implications of each one of the models previously described, and the possible consequences for the manipulation and design of electron-transfer pathways by protein engineering methods.

Theoretical treatments share the view that the amino acid matrix has been optimized naturally to guide electron tunneling and predict the selectability of each of the residues involved during protein evolution, perhaps even water molecules (49). In contrast, other models consider that the single characteristic selected for in the evolution of electron tunneling in redox-active enzymes is the positioning of redox centers within reach (140, 155). One interpretation of this position is that the flow of electrons transferred between redox centers does not necessarily follow a unique route, but that the shortest one will be favored for kinetic reasons. In consequence, every change of amino acid residue in the main route with deleterious effect on the electron transfer rate, will automatically elicit the engagement of alternative residues to form an equivalent secondary pathway.

Independent of the model supported, we consider that the use of proteins that naturally transfer electrons for hypothesis testing might present an inherent drawback. Electron transfer proteins, especially when associated with respiration, perform extremely important metabolic reactions. In this regard, they have been subject to millions years of pressure for any selectable trait: solubility, folding, thermal stability, rate, thermodynamic efficiency, *etc*. Perhaps the whole protein has been optimized for electron transfer. As an alternative, the feasibility of exploring the selectability of atypical electron transfer processes in redox-active proteins might shed some light on the discussion.

The oxidative inactivation of heme proteins is an unusual case of enzymatic performance where the enzyme itself is used as electron source and is led to irreversible deactivation as the oxidative damage accumulates (216). The molecular basis underlying this phenomenon is extraordinarily complex since the stoichiometric release of free radicals from the active site triggers the simultaneous occurrence of multiple reactions (216, 218). Independent of the source, protein-based free radicals travel inside the protein until their stabilization occurs at the position with the lowest redox potential available (92). The mechanism-based oxidative inactivation of heme peroxidases has been addressed from different standpoints, including directed evolution and site-directed mutagenesis, and the successful redox-inspired protein engineering strategy described below.

Significant results increasing oxidative stability in heme proteins have resulted from site-directed mutagenesis. In the case of cytochrome P450 BM-3, the substitution of F87A significantly increased the stability toward hydrogen peroxide (124). In hemoglobin, the formation of a stable thiyl radical decreased the rate of autoxidation and reduced heme degradation attributed to the reaction of superoxide with the heme (11).

Site-directed engineering of the hydrogen peroxide-binding pocket of a recombinant manganese peroxidase from *Phanerochaete chrysosporium* ATCC64314 allowed the recovery of a single methionine to leucine substitution that enhanced the tolerance of the enzyme towards low concentrations of hydrogen peroxide (1–3 m*M*) (137). In a subsequent report, selected positions inside the hydrogen peroxide-binding pocket of the same protein were subjected to saturation mutagenesis. Approximately 10,000 mutated proteins were individually synthesized in a cell-free expression system and screened for activity and stability. At the end of the screening procedure, 15 clones belonging to four different classes were sequenced. In all cases the common substitution was I83L; in addition A79 was changed for either E or S and N81 for S or L (138).

In the only example of molecular evolution of a heme protein aimed at increasing protein stability towards hydrogen

peroxide, Cherry and coworkers used a combination of approaches to develop a fungal peroxidase active in highly alkaline and oxidative conditions (40). Using the crystal structure as a guide, site-directed mutagenesis first targeted amino acid residues susceptible to oxidation by hydrogen peroxide. Three amino acids were identified and combined using site-directed mutagenesis to generate a variant with an oxidative stability fivefold higher than the wild-type enzyme and a thermal stability improvement of >100-fold. Further random mutagenesis and selection rounds identified a series of mutants with even further improvements in thermal stability and peroxide stability, but such improvements came at the cost of reducing the overall activity of the enzyme. To overcome this obstacle, the authors then shuffled *in vivo* clones with improved thermal stability with high activity clones. The output of these experiments was mutants with higher activity and higher stability than any of the input parental genes. A final round of *in vivo* shuffling using a pool of mutants with improved activity relative to the wild-type protein resulted in two distinct mutants with substitutions in the same position. The best of these was 174-times more thermally stable and 100-times more stable towards hydrogen peroxide than the starting point enzyme. Most of the changes selected in the former mutant were located inside the active site, mainly in the contact point between two helices which coordinate the binding of peroxide. The replacement of I49 to side chains able to establish hydrogen bonding was especially important. The I49S substitution increased oxidative stability 50-fold, probably by promoting the establishment of an alternative hydrogen bonding network.

With exception of the last example, where turnover rates were severely reduced, there was no indication if the mutation(s) were of any consequence on the catalytic properties of the enzymes. In all heme proteins, free radicals are produced with certain stoichiometry during catalytic turnover; in consequence, a drastic reduction on the catalytic parameters of the enzyme will pass for stability unless rigorously demonstrated.

We consider that only the redox-inspired reconfiguration of the electron abstraction pathways between the source and the alternative sinks would lead to productive electron-allocation equilibrium (216). Our experimental strategy assumed a large difference in redox potential between the heme iron atom and the rest of the protein components. The redox landscape of a protein can thus be deconvoluted from the usual representation (lineal or tridimensional) into a model that disregards the actual position or even the sequence of the amino acid residues in the polypeptide (Fig. 12). Most of the protein elements are redox neutral and are not considered in the model. Only elements, whether internal or external, in which redox value is different from the background are included. The actual number and identity of the elements depends on the presence of redox-active side chains, redox cofactors (organic or metallic), soluble electron donors/acceptors, and other proteins with a different global redox potential value.

Following these premises, we developed a redox-inspired strategy aimed towards remodeling the intramolecular electron transfer pathways involved in the oxidative deactivation of the model heme peroxidase c-type cytochrome from yeast (218). It is important to notice that these pathways are not the same as those which participate in the interprotein electron transfer between its physiological partners, and therefore have not been subject of selective pressure (102, 162). After several sequential substitution stages, a variant with a fully stable phenotype at catalytic concentrations of

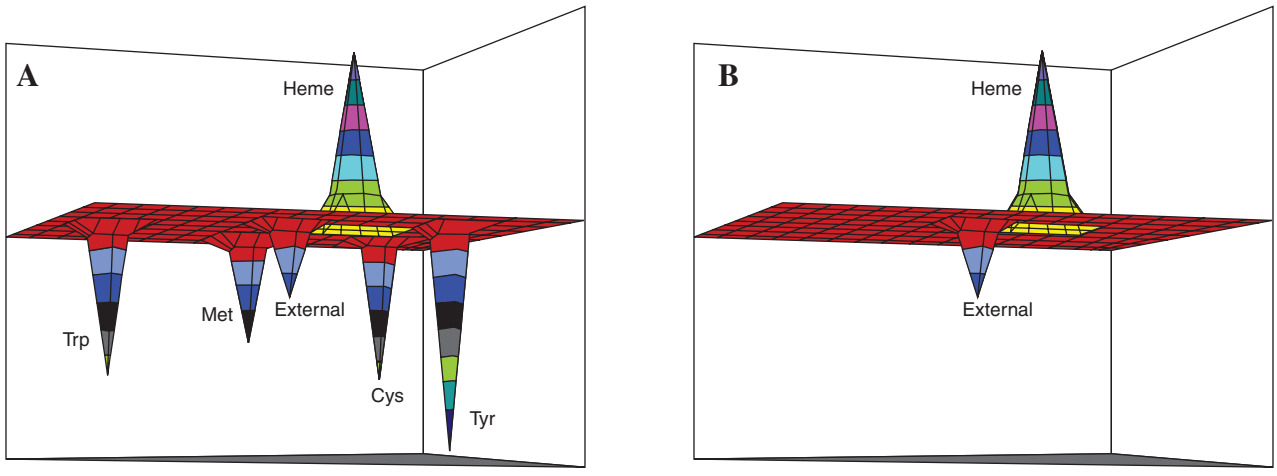

FIG. 12. Redox landscape of a model heme-protein. Ordinates represent the relative redox potential of electron acceptors with positive values and donors with negative values in arbitrary units. (**A**) The single peak with a positive value represents the activated heme-iron atom as the final electron sink (or free radical source). Peaks with negative values represent the external substrate and protein elements that may become potential electron sources (or free radical sinks). The external substrate behaves as a local minimum and, although it may allocate free radicals, it is out competed by the protein elements. The relative value of the external substrate minimum modulates the frequency with which it behaves as the electron donor, as has been demonstrated experimentally. (**B**) In the absence of low-redox potential side-chains, the external substrate behaves as the systemic minimum and will allocate all the free radicals produced during the enzymatic turnover, annulling the oxidation of the protein backbone and stabilizing the catalytic activity of the enzyme without compromise of the catalytic parameters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

hydrogen peroxide was obtained, harboring the N52I, W59F, Y67F, K79A, and F82G substitutions. None of these substitutions afforded stability separately or even in smaller groups, indicating that disruption of electron transfer from the multiple protein donors to the iron acceptor must be absolute. The phenotype of this variant was reflected in the stability of its electronic components, allowing the identification of a protein-based radical intermediate.

Several lines of evidence indicated that the internal electron donors were tyrosine residues (168, 223). Simultaneous substitution of the five positions abolished the formation of the protein-based radical signal, supporting their suggested role. The radical was still formed and was located as a transient porphyrin-based species ($g_{\parallel} = 2.52$ and $g_{\perp} = 2.0$ values), indicating that in the absence of tyrosine side chains, the porphyrin moiety acted as the final electron source (217). Interestingly, removal of the tyrosine residues did not improve the stability of the protein on the contrary, the tyrosine-less mutant was 10 times less competent to use external donors. This phenotype indicates that other protein elements were still used as electron donors, although the redox potential difference with the oxo-iron(IV)porphyrin acceptor was less significant.

Comparison of these experiments allows us to draw several conclusions: (a) If there was a major electron/radical transfer pathway from the donor sites to the acceptor, it would have been exposed after several rounds of random mutagenesis and stringent selection. Protein perturbation was intense enough to generate variants that mimicked the selected phenotype without alteration of the electron transfer process. However, electron transfer and oxidative damage accumulations were not affected in the mutants. The phenotype is consequence of a strong reduction on the radical production rate. Macroscopically, the effect is similar, i.e., acquired resistance to oxidative deactivation. (b) Development of a rational approach that assumed the absence of major electron transfer pathways yielded positive results after the "electric isolation" of the acceptor from possible protein donors.

From this evidence we support the view that most of the primary sequence of the protein is irrelevant for electron transfer, and that the chemical properties of the residues involved in the pathways are less critical than other factors (45).

VII. Conclusions

This review serves several purposes. One of them is to give an updated account of the methods used for protein engineering aimed at non experts. In order to offer a comprehensive view of the field, we decided to include both genetic and chemical methods which are usually reviewed separately. Specific applications to redox-active enzymes are mentioned along each technology, with emphasis in those cases where the generation of novel functionality was pursued. Finally, we focused in two emerging fields in the protein engineering of redox-active enzymes: the construction of novel nucleic acid-based catalysts and the remodeling of intramolecular electron transfer networks. We consider that the future development of these areas will represent fine examples of the concurrence of chemical and genetic tools. In conclusion, with the advent of the powerful tools here described, this seems to be an excellent time for projects based on the protein engineering of redox-active enzymes.

Acknowledgments

We are indebted to Dr. Juan Téllez for his support in figure preparation and to Professors Michael A. Pickard and Juan Miranda for a critically reading of the manuscript. GS acknowledges the support of CONACYT p49590-Q and BV of PAPIIT IN202407-3 and CONACYT 50581.

Abbreviations

BLAST, basic local alignment search tool; CAST, Combinatorial Active-site Saturation Test; CTQ, cysteine-tryptophyl-quinone; DHFR, dihydrofolate reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GMPS, guanosine-5′-monophosphorothioate; LTQ, lysine tyrosylquinone; MBD, metalloprotein database and browser; NAD, nicotinamide adenine dinucleotide; PCR, polymerase chain reaction; PDB, protein database; PQQ, pyrroloquinoline quinine; RACHITT, random chimeragenesis on transient templates; RNR, class I ribonucleotide reductase; SCHEMA, structure-based CHEMeragensis algorithm; SHIPREC, sequence homology-independent protein recombination; TPQ, topaquinone; TTQ, tryptophan-tryptophyl-quinone; VAO, vanillil-alcohol oxidase.

References

1. Alfonta L, Zhang Z, Uryu S, Loo JA, and Schultz PG. Site-specific incorporation of a redox-active amino acid into proteins. *J Am Chem Soc* 125: 14662–14663, 2003.
2. Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ. Basic local alignment search tool. *J Mol Biol* 215: 403–410, 1990.
3. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl Acids Res* 25: 3389–3402, 1997.
4. Andreini C, Banci L, Bertini I, Elmi S, and Rosato A. Non-heme iron through the three domains of life. *Proteins* 67: 317–324, 2007.
5. Andreini C, Banci L, Bertini I, and Rosato A. Counting the zinc-proteins encoded in the human genome. *J Proteome Res* 5: 196–201, 2006.
6. Anthony C. Quinoprotein-catalysed reactions. *Biochem J* 320:697–711, 1996.
7. Anthony C. Methanol dehydrogenase, a PQQ-containing quinoprotein dehydrogenase. In: *Enzyme-Catalyzed Electron and Radical Transfer*, edited by Scrutton NS and Holzenburg A. New York, NY: Kluwer Academic Publishers, 2002, p. 73–117
8. Anthony C. The quinoprotein dehydrogenases from methanol and glucose. *Arch Biochem Biophys* 428: 2–9, 2004.
9. Axarli I, Prigipaki A, and Labrou NE. Engineering the substrate specificity of cytochrome P450 CYP102A2 by directed evolution: production of an efficient enzyme for bioconversion of fine chemicals. *Biomol Engin* 22: 81–88, 2005.
10. Bain JD, Glabe CG, Dix TA, Chamberlin AR, and Siala ES. Biosynthetic site-specific incorporation of a non-natural amino acid into a polypeptide. *J Am Chem Soc* 111: 8013–8014, 1989.
11. Balagopalakrishna C, Abugo OO, Horsky J, Manoharan PT, Nagababu E, and Rifkind JM. Superoxide produced in the

heme pocket of the beta-chain of hemoglobin reacts with the beta-93 cysteine to produce a thiyl radical. *Biochemistry* 37: 13194–13202, 1998.
12. Bang D and Kent SBH. His6 tag-assisted chemical protein synthesis. *Proc Natl Acad Sci USA* 102: 5014, 2005.
13. Becker CFW, Lausecker K, Balog M, Kalai T, Hideg K, Steinhoff HJ, and Engelhard M. Incorporation of spin-labelled amino acids into proteins. *Magn Reson Chem* 43: S34–S39, 2005.
14. Beligere GS and Dawson PE. Synthesis of a three zinc finger protein, Zif268, by native chemical ligation. *Biopolymers* 51: 363–369, 1999.
15. Bennati M, Robblee JH, Mugnaini V, Stubbe J, Freed JH, and Borbat P. EPR distance measurements support a model for long-range radical initiation in E. coli ribonucleotide reductase. *J Am Chem Soc* 127: 15014–15015, 2005.
16. Benner SA, Ellington AD, and Tauer A. Modern metabolism as a palimpsest of the RNA world. *Proc Natl Acad Sci USA* 86: 7054–7058, 1989.
17. Benson DE, Wisz MS, and Hellinga HW. The development of new biotechnologies using metalloprotein design. *Curr Opin Biotechnol* 9: 370–376, 1998.
18. Benson DE, Wisz MS, and Hellinga HW. Rational design of nascent metalloenzymes. *Proc Natl Acad Sci USA* 97: 6292–6297, 2000.
19. Beratan DN, Betts JN, and Onuchic JN. Protein electron transfer rates set by the bridging secondary and tertiary structure. *Science* 252: 1285–1288, 1991.
20. Beratan DN, Onuchic JN, and Hopfield JJ. Electron tunneling through covalent and noncovalent pathways in proteins. *J Chem Phys* 86: 4488–4498. 1987.
21. Berg JM. Zinc finger domains: From predictions to design. *Acc Chem Res* 28: 14–19, 1995.
22. Bershtein S, Segal M, Bekerman R, Tokuriki N, and Tawfik DS. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. *Nature* 444: 929–932, 2006.
23. Bertini I, Gray HB, Lippard SJ, and Valentine JS. *Bioinorganic chemistry*. Mill Valley, CA: University Science Books, 1994.
24. Bertini I and Rosato A. From genes to metalloproteins: A bioinformatic approach. *Eur J Inorg Chem* 18:2546–2555, 2007.
25. Bloom JD, Arnold FH, and Wilke CO. Breaking proteins with mutations: threads and thresholds in evolution. *Mol Sys Biol* 3: 76, 2007.
26. Bocola M. Directed evolution of enantioselective enzymes: Iterative cycles of CASTing for probing protein-sequence space. *Angew Chem Int Ed* 45: 1236–1241, 2006.
27. Bornscheuer UT and Pohl M. Improved biocatalysts by directed evolution and rational protein design. *Curr Opin Chem Biol* 5: 137–143, 2001.
28. Breaker RR. In vitro selection of catalytic polynucleotides. *Chem Rev* 97: 371–390, 1997.
29. Breaker RR and Joyce GF. Self-incorporation of coenzymes by ribozymes. *J Mol Evol* 40: 551–558, 1995.
30. Bugg TDH and Ramaswamy S. Non-heme iron-dependent dioxygenases: unravelling catalytic mechanisms for complex enzymatic oxidations. *Curr Opinion Chem Biol* 12: 134–140, 2008.
31. Bulter T, Alcalde M, Sieber V, Meinhold PP, Schlachtbauer Ch, and Arnold FH. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Appl Environ Microbiol* 69: 987–995, 2003.
32. Burgstaller P and Famulok M. Isolation of RNA aptamers for biological cofactors by in vitro selection. *Angew Chem Int Ed* (*English*) 33: 1084–1087, 1994.
33. Cadwell RC and Joyce GF. Mutagenic PCR. *PCR Methods Appl* 3: S136–S140, 1994.
34. Canters GW and Dennison C. Biological electron transfer: structural and mechanistic studies. *Biochimie* 77: 506–515, 1995.
35. Capozzi F, Ciurli S, and Luchinat C. Coordination sphere versus protein environment as determinants of electronic and functional properties of iron-sulfur proteins. *Struct Bond* 90: 127–160, 1998.
36. Castagnetto JM, Hennessy SW, Roberts VA, Getzoff ED, Tainer JA, and Pique ME. MDB: The metalloprotein database and browser at the Scripps Research Institute. *Nucl Acids Res* 30: 379–382, 2002.
37. Chang MC, Yee CS, Nocera DG, and Stubbe J. Site-specific replacement of a conserved tyrosine in ribonucleotide reductase with an aniline amino acid: a mechanistic probe for a redox-active tyrosine. *J Am Chem Soc* 126: 16702–16703, 2004.
38. Cheddar G, Meyer TE, Cusanovich MA, Stout CD, and Tollin G. Redox protein electron-transfer mechanisms: electrostatic interactions as a determinant of reaction site in *c*-type cytochromes. *Biochemistry* 28: 6318–6322, 1989.
39. Chen SY, Cressman S, Mao F, Shao H, Low DW, Beilan HS, Cagle EN, Carnevali M, Gueriguian V, Keogh PJ, Porter H, Stratton SM, Wiedeke MC, Savatski L, Adamson JW, Bozzini CE, Kung A, Kent SBH, Bradburne JA, and Kochendoerfer GG. Synthetic erythropoietic proteins: Tuning biological performance by site-specific polymer attachment. *Chem Biol* 12: 371–383, 2005.
40. Cherry JR, Lamsa MH, Schneider P, Vind J, Svendsen A, Jones A, and Pedersen AH. Directed evolution of a fungal peroxidase. *Nature Biotech* 17: 379–384, 1999.
41. Claus J, Andrea LH, and Fiona HF. The sulfinic acid switch in proteins. *Org Biomol Chem* 2: 1953–1956, 2004.
42. Clouthier CM, Kayser MM, and Reetz MT. Designing new Baeyer–Villiger monooxygenases using restricted CASTing. *J Org Chem* 71: 8431–8437, 2006.
43. Cochran FV, Wu SP, Wang W, Nanda V, Saven JG, Therien MJ, and DeGrado WF. Computational *de novo* design and characterization of a four-helix bundle protein that selectively binds a nonbiological factor. *J Am Chem Soc* 127: 1346–1347, 2005.
44. Coco WM, Levinson WE, Crist MJ, Hektor HJ, Darzins A, Pienkos PT, Squires CH, and Monticello DJ. DNA shuffling method for generating highly recombined genes and evolved enzymes. *Nature Biotech*. 19: 354–359, 2001.
45. Coppock DL and Thorpe C. Multidomain flavin-dependent sulfhydryl oxidases. *Antioxid Redox Signal* 8: 300–311, 2006.
46. Crameri A, Raillard SA, Bermudez E, and Stemmer WP. DNA shuffling of a family of genes from diverse species accelerates directed evolution. *Nature* 391: 288–291, 1998.
47. Creighton T.E. The folded conformation of globular proteins. In: *Proteins. Structure and Molecular Properties* New York, NY: W.H. Freeman and Company, 1993, p. 202–260.
48. Dai QH, Tommos C, Fuentes EJ, Blomberg MRA, Dutton PL, and Wand AJ. Structure of a de novo designed protein model of radical enzymes. *J Am Chem Soc* 124: 10952–10953, 2002.
49. Daizadeh I, Medvedeved DM, and Stuchebrukhov AA. Electron transfer in ferrodoxin: Are tunneling pathways evolutionarily conserved? *Mol Biol Evol* 19: 406–415, 2002.
50. Datta S, Mori Y, Takagi K, Kawaguchi K, Chen ZW, Okajima T, Kuroda S, Ikeda T, Kano K, and Tanizawa K. Structure of a quinohemoprotein amine dehydrogenase with an

uncommon redox cofactor and highly unusual crosslinking. *Proc Natl Acad Sci USA* 241429098, 2001.
51. Datta S, Mori Y, Takagi K, Kawaguchi K, Chen ZW, Okajima T, Kuroda S, Ikeda T, Kano K, Tanizawa K, and Mathews FS. Structure of a quinohemoprotein amine dehydrogenase with an uncommon redox cofactor and highly unusual crosslinking. *Proc Natl Acad Sci USA* 98: 14268–14273, 2001. (Reference deleted at the galley proof stage.)
52. Davidson VL. What controls the rates of interprotein electron-transfer reactions. *Acc Chem Res* 33: 87–93, 2000.
53. Davidson VL. Electron transfer in quinoproteins. *Arch Biochem Biophys* 428: 32–40, 2004.
54. Davies MJ. The oxidative environment and protein damage. *Biochim Biophys Acta* 1703: 93–109, 2005.
55. Dawson PE and Kent SBH. Synthesis of native proteins by chemical ligation. *Annu Rev Biochem* 69: 923–960, 2000.
56. Dawson PE, Muir TW, Clark-Lewis I, and Kent SB. Synthesis of proteins by native chemical ligation. *Science* 266: 776–779, 1994.
57. Degtyarenko KN, North ACT, and Findlay JBC. PROMISE: a database of bioinorganic motifs. *Nucl Acids Res* 27: 233–236, 1999.
58. Dokmanic I, Sikic M, and Tomic S. Metals in proteins: correlation between the metal-ion type, coordination number and the amino-acid residues involved in the coordination. *Acta Crystallogr D Biol Crystallogr* D64: 257–263, 2008.
59. Dooley DM. Structure and biogenesis of topaquinone and related cofactors. *J Biol Inorg Chem* 4: 1–11, 1999.
60. Duine JA. Quinoproteins: enzymes containing the quinonoid cofactor pyrroloquinoline quinone, topaquinone or tryptophan-tryptophanquinone. *Eur J Biochem* 200: 271–284, 1991.
61. Duine JA. The PQQ history. *J Biosc Bioeng* 88: 231–236, 1999.
62. Durek T and Becker CFW. Protein semi-synthesis: New proteins for functional and structural studies. *Biomol Eng* 22: 153–172, 2005.
63. Durek T, Torbeev VY, and Kent SBH. Convergent chemical synthesis and high-resolution x-ray structure of human lysozyme. *Proc Natl Acad Sci USA* 104: 4846, 2007.
64. Ebert JC and Altman RB. Robust recognition of zinc binding sites in proteins. *Prot Sci* 17: 54–65, 2008.
65. Eckenroth B, Harris K, Turanov AA, Gladyshev VN, Raines RT, and Hondal RJ. Semisynthesis and characterization of mammalian thioredoxin reductase. *Biochemistry* 45: 5158–5170, 2006.
66. Eidsness MK, Burden AE, Richie KA, Kurtz DM, Scott RA, Smith ET, Ichiye T, Beard B, Min TP, and Kang CH. Modulation of the redox potential of the (Fe(SCys)(4)) site in rubredoxin by the orientation of a peptide dipole. *Biochemistry* 38: 14803–14809, 1999.
67. Ekberg M, Sahlin M, Eriksson M, and Sjoberg BM. Two conserved tyrosine residues in protein R1 participate in an intermolecular electron transfer in ribonucleotide reductase. *J Biol Chem* 271: 20655–20659, 1996.
68. England PM. Unnatural amino acid mutagenesis: A precise tool for probing protein structure and function. *Biochemistry* 43: 11623–11629, 2004.
69. Ergenekan CE, Thomas D, Fischer JT, Tan ML, Eidsness MK, Kang CH, and Ichiye T. Prediction of reduction potential changes in rubredoxin: A molecular mechanics approach. *Biophys J* 85: 2818–2829, 2003.
70. Evans Jr TC, Benner J, and Xu MQ. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Prot Sci* 7: 2256–2264, 1998.
71. Farver O, Skov LK, van de Kamp M, Canters GW, and Pecht I. The effect of driving force on intramolecular electron transfer in proteins. Studies on single-site mutated azurins. *Eur J Biochem* 210: 399–403, 1992.
72. Felsenstein J. The evolutionary advantage of recombination. *Genetics* 78: 737–756, 1974.
73. Felsenstein J and Yokoyama S. The evolutionary advantage of recombination. II. Individual selection for recombination. *Genetics* 83: 845–859, 1976.
74. Fiser A and Simon I. Predicting the oxidation state of cysteines by multiple sequence alignment. *Bioinformatics* 16: 251–256, 2000.
75. Flores H and Ellington A.D. A modified consensus approach to mutagenesis inverts the cofactor specificity of *Bacillus stearothermophilus* lactate dehydrogenase. Oxford U Press; *Prot Engi Design Selection* 18: 369–377. 2005.
76. Fomenko DE and Gladyshev VN. CxxS: Fold-independent redox motif revealed by genome-wide searches for thiol/disulfide oxidoreductase function. *Prot Sci* 11: 2285–2296, 2002.
77. Fomenko DE and Gladyshev VN. Identity and functions of CxxC-derived motifs. *Biochemistry* 42: 11214–11225, 2003.
78. Fomenko DE, Xing W, Adair BM, Thomas DJ, and Gladyshev VN. High-throughput identification of catalytic redox-active cysteine residues. *Science* 315: 387–389, 2007.
79. Futaki S, Tatsuto K, Shiraishi Y, and Sugiura Y. Total synthesis of artificial zinc-finger proteins: Problems and perspectives. *Biopolymers* 76: 98–109, 2004.
80. Gaertner HF, Offord RE, Cotton R, Timms D, Camble R, and Rose K. Chemo-enzymic backbone engineering of proteins. Site-specific incorporation of synthetic peptides that mimic the 64-74 disulfide loop of granulocyte colony-stimulating factor. *J Biol Chem* 269: 7224–7230, 1994.
81. Gaertner HF, Rose K, Cotton R, Timms D, Camble R, and Offord RE. Construction of protein analogs by site-specific condensation of unprotected fragments. *Bioconjugate Chem* 3: 262–268, 1992.
82. Gaytán P, Yañez J, Sánchez F, Mackie H, and Soberón X. Combination of DMT-mononucleotide and Fmoc-trinucleotide phosphoramidites in oligonucleotide synthesis affords an automatable codon-level mutagenesis method. *Chem Biol* 5: 519–527, 1998.
83. Giles NM, Giles GI, and Jacob C. Multiple roles of cysteine in biocatalysis. *Biochem Biophys Res Com* 300: 1–4, 2003.
84. Goodwin PM and Anthony C. The biochemistry, physiology and genetics of PQQ and PQQ-containing enzymes. *Adv Microb Physiol* 40: 1–80, 1998.
85. Gray HB and Winkler JR. Electron transfer in proteins. *Annu Rev Biochem* 65: 537–561, 1996.
86. Gray HB and Winkler JR. Electron tunneling through proteins. *Quart Rev Biophys* 36: 341–372, 2004.
87. Gregory DS, Martin ACR, Cheetham JC, and Rees AR. The prediction and characterization of metal binding sites in proteins. *Prot Eng* 6: 29–35, 1993.
88. Guzmán F, Barberis S, and Illanes A. Peptide synthesis: chemical or enzymatic. *Elect J Biotechnol* 10: 279–314, 2007.
89. Hahn ME and Muir TW. Manipulating proteins with chemistry: a cross-section of chemical biology. *Trends Biochem Sci* 30: 26–34, 2005.
90. Hall AN and Tomsett AB. Structure-function analysis of NADPH: nitrate reductase from *Aspergillus nidulans*: Analysis of altered pyridine nucleotide specificity in vivo. *Microbiology* 146: 1399–1406, 2000.

91. Harding MM. Geometry of metal-ligand interactions in proteins. *Acta Crystallogr D Biol Crystallogr* 57: 401–411, 2001.
92. Hawkins CL and Davies MJ. Generation and propagation of radical reactions on proteins. *Biochim Biophys Acta* 1504: 196–219, 2001.
93. Hay S, Westerlund K, and Tommos C. Redox characteristics of a de novo quinone protein. *J Phys Chem* 111: 3488–3495, 2007.
94. Hecht MH, Das A, Go A, Bradley LH, and Wei Y. De novo proteins from designed combinatorial libraries. *Prot Sci* 13: 1711–1723, 2004.
95. Held DM, Travis Greathouse S, Agrawal A, and Burke DH. Evolutionary landscapes for the acquisition of new ligand recognition by RNA aptamers. *J Mol Evol* 57: 299–308, 2003.
96. Hellinga HW and Richards FM. Construction of new ligand binding sites in proteins of known structure. I. Computer-aided modeling of sites with pre-defined geometry. *J Mol Biol* 222: 763–785, 1991.
97. Holm RH, Kennepohl P, and Solomon EI. Structural and functional aspects of metal sites in biology. *Chem Rev* 96: 2239–2314, 1996.
98. Hondal RJ. Incorporation of selenocysteine into proteins using peptide ligation. *Prot Peptide Lett* 12: 757–764, 2005.
99. Huang F, Bugg CW, and Yarus M. RNA-catalyzed CoA, NAD, and FAD synthesis from phosphopantetheine, NMN, and FMN. *Biochemistry* 39: 15548–15555, 2000.
100. Huang F and Yarus M. Versatile 5′ phosphoryl coupling of small and large molecules to an RNA. *Proc Natl Acad Sci USA* 94:8965–8969, 1997.
101. Huang YW, Pineau I, Chang HJ, Azzi A, Bellemare V, Laberge S, and Lin SX. Critical residues for the specificity of cofactors and substrates in human estrogenic 17β-hydroxysteroid dehydrogenase 1: Variants designed from the three-dimensional structure of the enzyme. *Mol Endocrinol* 15: 2010–2020, 2001.
102. Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S, and Jap BK. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. *Science* 281: 58–59, 1998.
103. Jacob C, Giles GI, Giles NM, and Sies H. Sulfur and selenium: The role of oxidation state in protein structure and function. *Agnew Chem Int Ed* 42: 4742–4758, 2003.
104. Jadhav VR and Yarus M. Coenzymes as coribozymes. *Biochimie* 84: 877–888, 2002.
105. Johansson JS, Gibney BR, Skalicky JJ, Wand AJ, and Dutton PL. A native-like three-alpha-helix bundle protein from structure-based redesign: A novel maquette scaffold. *J Am Chem Soc* 120: 3881–3886, 1998.
106. Johnson ECB and Kent SBH. Towards the total chemical synthesis of integral membrane proteins: a general method for the synthesis of hydrophobic peptide-(alpha)thioester building blocks. *Tetrahedron Lett* 48: 1795–1799, 2007.
107. Joosten V and van Berkel WJH. Flavoenzymes. *Curr Opin Chem Biol* 11: 195–202, 2007.
108. Kaplan J and DeGrado WF. *De novo* design of catalytic proteins. *Proc Natl Acad Sci USA* 101: 11566–11570, 2004.
109. Kawatsu T, Kakitani T, and Yamato T. Worm model for electron tunneling in proteins: Consolidation of the pathway model and the Dutton plot. *J Phys Chem B*. 105: 4424–4435, 2001.
110. Kennedy ML and Gibney BR. Metalloprotein and redox protein design. *Curr Opin Struct Biol* 11: 485–490, 2001.
111. Kimmerlin T and Seebach D. '100 years of peptide synthesis': ligation methods for peptide and protein synthesis with applications to b-peptide assemblies. *J Peptide Res* 65: 229–260, 2005.
112. Kimmerlin T, Seebach D, and Hilvert D. Synthesis of β3-peptides and mixed α/β3-peptides by thioligation. *Helv Chim Acta* 85: 1812–1826, 2002.
113. Klinman JP. New quinocofactors in eukayotes. *J Biol Chem* 271: 27189–27192, 1996.
114. Kochendoerfer GG, Clayton D, and Becker C. Chemical synthesis approaches to the engineering of ion channels. *Prot Peptide Lett* 12: 737–741, 2005.
115. Kochendoerfer GG and Kent SBH. Chemical protein synthesis. *Curr Opinion Chem Biol* 3: 665–671, 1999.
116. Koder RL and Dutton L. Intelligent design: the de novo engineering of proteins with specified functions. *Dalton Trans* 25: 3045–3051, 2006.
117. Koski LB and Golding GB. The closest BLAST hit is often not the nearest neighbor. *J Mol Evol* 52: 540–542, 2001.
118. Kumamaru T, Suenaga H, Mitsuoka M, Watanabe T, and Furukawa K. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. *Nature Biotech* 16: 617–618, 1998.
119. Kumar RA, Koc A, Cerny RL, and Gladyshev VN. Reaction mechanism, evolutionary analysis, and role of zinc in *Drosophila* methionine-R-sulfoxide reductase. *J Biol Chem* 277: 37527–37535, 2002.
120. Kumar S, Chen CS, Waxman DJ, and Halpert JR. Directed evolution of mammalian cytochrome P450 2B1: Mutations outside of the active site enhance the metabolism of several substrates, including the anticancer prodrugs cyclophosphamide and ifosfamide. *J Biol Chem* 280: 19569–19575, 2005.
121. Landwehr M, Carbone M, Otey CR, Li Y, and Arnold FH. Diversification of catalytic function in a synthetic family of chimeric cytochrome P450s. *Chem Biol* 14: 269–278, 2007.
122. Langen R, Chang IJ, Germanas JP, Richards JH, Winkler JR, and Gray HB. Electron tunneling in proteins—coupling through a beta strand. *Science* 268: 1733–1735, 1995.
123. Lauhon CT and Szostak JW. RNA aptamers that bind flavin and nicotinamide redox cofactors. *J Am Chem Soc* 117: 1246–1257, 1995.
124. Li QS, Ogawa J, and Shimizu S. Critical role of the residue size at position 87 in H_2O_2-dependent substrate hydroxylation activity and H_2O_2 inactivation of cytochrome P450BM-3. *Biochem Biophys Res Commun* 280: 1258–1261, 2001.
125. Li Y, Drummond DA, Sawayama AM, Snow CD, Bloom JD, and Arnold FH. A diverse family of thermostable cytochrome P450s created by recombination of stabilizing fragments. *Nature Biotech* 25: 1051, 2007.
126. Liang L, Zhang J, and Lin Z. Altering coenzyme specificity of *Pichia stipitis* xylose reductase by the semi-rational approach CASTing. *Micro Cell Fact* 6: 36, 2007.
127. Lin J, Balabin IA, and Beratan DN. The nature of aqueous tunneling pathways between electron-transfer proteins. *Science* 310: 1311–1313, 2005.
128. Link AJ, Mock ML, and Tirrell DA. Non-canonical amino acids in protein engineering. *Curr Opin Biotech* 14: 603–609, 2003.
129. Lisdat F, Ge B, Krause B, Ehrlich A, Bienert H, and Scheller FW. Nucleic acid promoted electron transfer to cytochrome c. *Electroanalysis* 13: 1225, 2001.
130. Liu DR, Magliery TJ, Pastrnak M, and Schultz PG. Engineering a tRNA and aminoacyl-tRNA synthetase for the

site-specific incorporation of unnatural amino acids into proteins *in vivo*. *Proc Natl Acad Sci USA* 94: 10092–10097, 1997.
131. Lorsch JR and Szostak JW. *In vitro* selection of RNA aptamers specific for cyanocobalamin. *Biochemistry* 33: 973–982, 1994.
132. Marcus RA and Sutin N. Electron transfer in chemistry and biology. *Biochim Biophys Acta* 811: 265–322, 1985.
133. Mathews FC, Cunane L, and Durley RCE. Flavin electron transfer proteins. In: *Enzyme-Catalyzed Electron and Radical Transfer*, edited by Scrutton NS and Holzenburg A. New York, NY: Kluwer Academic Publishers, 2002, p. 29–72
134. McIntire WS, Wemmer DE, Chistoserdov A, and Lidstrom ME. A new cofactor in a prokaryotic enzyme: tryptophan tryptophylquinone as the redox prosthetic group in methylamine dehydrogenase. *Science* 252: 817–824, 1991.
135. Meinhold P, Peters MW, Chen MMY, Takahasi K, and Arnold FH. Direct conversion of ethane to ethanol by engineered cytochrome P 450 BM 3. *Chem Bio Chem (Print)* 6: 1765–1768, 2005.
136. Mills DR, Peterson RL, and Spiegelman S. An extracellular Darwininan experiment with a self-duplicating nucleic acid molecule. *Proc Natl Acad Sci USA* 58: 217–224, 1967.
137. Miyazaki C and Takahashi H. Engineering of the H2O2-binding pocket region of a recombinant manganese peroxidase to be resistant to H2O2. *FEBS Lett* 509: 111–114, 2001.
138. Miyazaki-Imamura C, Oohira K, Kitagawa R, Nakano H, Yamane T, and Takahashi H. Improvement of H2O2 stability of manganese peroxidase by combinatorial mutagenesis and high-throughput screening using *in vitro* expression with protein disulfide isomerase. *Protein Eng* 16: 423–428, 2003.
139. Moser CC, Keske JM, Warncke K, Farid RS, and Dutton PL. Nature of biological electron transfer. *Nature* 355: 796–802, 1992.
140. Moser CC, Page CC, and Dutton PL. Darwin at the molecular scale: selection and variance in electron tunnelling proteins including cytocromo *c* oxidase. *Philos Trans Royal Soc B: Biol Sci* 361: 1295–1305, 2006.
141. Muir TW, Sondhi D, and Cole PA. Expressed protein ligation: a general method for protein engineering. *Proc Natl Acad Sci USA* 95: 6705–6710, 1998.
142. Muir TW. Semisynthesis of proteins by expressed protein ligation. *Annu Rev Biochem* 72: 249–289, 2003.
143. Muralidharan V and Muir TW. Protein ligation: An enabling technology for the biophysical analysis of proteins. *Nature Meth* 3: 429–438, 2006.
144. Muratovska A, Lightowlers RN, Taylor RW, Turnbull DM, Smith RAJ, Wilce JA, Martin SW, Murphy MP, and Journals O. Targeting peptide nucleic acid (PNA) oligomers to mitochondria within cells by conjugation to lipophilic cations: Implications for mitochondrial DNA replication, expression and disease. *Nucl Acids Res* 29: 1852–1863, 2001.
145. Mure M, Mills SA, and Klinman JP. Catalytic mechanism of the topa quinone containing copper amine oxidases. *Biochemistry* 41: 9269–9278, 2002.
146. Mutter M, Hersperger R, Gubernator K, and Muller K. The construction of new proteins: V. A template-assembled synthetic protein (TASP) containing both a 4-helix bundle and beta-barrel-like structure. *Prot Str Function Genet* 5: 13–21, 1989.
147. Nilsson BL, Soellner MB, and Raines RT. Chemical synthesis of proteins. *Annu Rev Biophys Biomol Struct* 34: 91–118, 2005.
148. Noren CJ, Anthony-Cahill SJ, Griffith MC, and Schultz PG. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* 244: 182–188, 1989.
149. Noren CJ, Wang J, and Perler FB. Dissecting the chemistry of protein splicing and its applications. *Angew Chem Int Ed Engl* 39: 450–466, 2000.
150. Offord RE. Protection of peptides of biological origin for use as intermediates in the chemical synthesis of proteins. *Nature* 221: 37, 1969.
151. Offord, R. E. Protein semisynthesis in theory and practice. In: *Semisynthetic Peptides and Proteins*, edited by Offord RE and Di Bello C. London: Academic Press, 1978, p. 3.
152. Onuchic JN, Beratan DN, Winkler JR, and Gray HB. Pathway analysis of protein electron-transfer reactions. *Annu Rev Biophys Biomol Struct* 21: 349–377, 1992.
153. Orengo CA, Michie AD, Jones S, Jones DT, Swindells MB, and Thornton JM. CATH–-a hierarchic classification of protein domain structures. *Structure* 5: 1093–1108, 1997.
154. Otey CR, Landwehr M, Endelman JB, Hiraga K, Bloom JD, and Arnold FH. Structure-guided recombination creates an artificial family of cytochromes P450. *PLoS Biology* 4: e112, 2006.
155. Page CC, Moser CC, Chen X, and Dutton PL. Natural engineering principles of electron tunnelling in biological oxidation-reduction. *Nature* 402: 47–52, 1999.
156. Palmer AE, Szilagyi RK, Cherry JR, Jones A, Xu F, and Solomon E. I. Spectroscopic characterization of the Leu513His variant of fungal laccase: Effect of increased axial type ligand interaction on the geometric and electronic structure of the Type 1 Cu site. *J Inorg Chem* 42: 4006–4017. 2003.
157. Panmanee W, Vattanaviboon P, Poole LB, and Mongkolsuk S. Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *J Bacteriol* 188: 1389–1395, 2006.
158. Passerini A, Andreini C, Menchetti S, Rosato A, and Frasconi P. Predicting zinc binding at the proteome level. *BMC Bioinformatics* 8: 39, 2007.
159. Pasternak A, Kaplan J, Lear JD, and DeGrado WF. Proton and metal ion-dependet assembly of a model diiron protein. *Prot Sci* 10: 958–969, 2001.
160. Pattnaik SB and Suga H. Reengineering aninvitroevolved ribozyme. *Nucl Acids Symp Series* 51: 381, 2007.
161. Paul N, Springsteen G, and Joyce GF. Conversion of a ribozyme to a deoxyribozyme through *in vitro* evolution. *Chem Biol* 13: 329–338, 2006.
162. Pelletier H and Kraut J. Crystal structure of a complex between electron transfer partners, cytochrome c peroxidase and cytochrome c. *Science* 258: 1748–1755, 1992.
163. Pellois JP and Muir TW. Semisynthetic proteins in mechanistic studies: using chemistry to go where nature can't. *Curr Opinion Chem Biol* 10: 487–491, 2006.
164. Perler FB. InBase: the Intein database. *Nucl Acids Res* 30: 383, 2002.
165. Peters MW, Meinhold P, Glieder A, and Arnold FH. Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3. *J Am Chem Soc* 125: 13442–13450, 2003.
166. Pierre JL and Fontecave M. Iron and activated oxygen species in biology: the basic chemistry. *Biometals* 12: 195–199, 1999.
167. Poole LB, Karplus PA, and Claiborne A. Protein sulfenic acids in redox signaling. *Ann Rev Pharmacol Toxicol* 44: 325–347, 2004.

168. Qian SY, Chen Y-R, Deterding LJ, Fann YC, Chignell CF, Tomer KB, and Mason RP. Identification of protein-derived tyrosyl radical in the reaction of cytochrome c and hydrogen peroxide: characterization by ESR spin-trapping, HPLC and MS. *Biochem J* 363: 281–288, 2002.
169. Rackman O and Chin JW. A network of orthogonal ribosome-mRNA pairs. *Nature Chem Biol* 1: 159–166, 2005.
170. Rackman O and Chin JW. Cellular logic with orthogonal ribosomes. *J Am Chem Soc* 127: 17584–17585, 2005.
171. Reece SY, Hodgkiss JM, Stubbe JA, and Nocera DG. Proton-coupled electron transfer: the mechanistic underpinning for radical transport and catalysis in biology. *PhilosTrans Royal Soc B: Biol Sci* 361: 1351–1364, 2006.
172. Rees DC. Great metalloclusters in enzymology. *Annu Rev Biochem* 71:221-246, 2002.
173. Rees DC and Howard JB. The interface between the biological and inorganic worlds: Iron–sulfur metalloclusters. *Science* 300: 929–931, 2003.
174. Reetz MT, Carballeira JD, Peyralans J, Hobenreich H, Maichele A, and Vogel A. Expanding the Substrate Scope of Enzymes: Combining Mutations Obtained by CASTing. *space* 17:19, (Reference deleted at the galley proof stage.)
175. Reetz MT, Carballeira JD, Peyralans J, Hobenreich H, Maichele A, and Vogel A. Expanding the substrate scope of enzymes: combining mutations obtained by CASTing. *Chemistry* 12: 6031–6038, 2006.
176. Reetz MT, Torre C, Eipper A, Lohmer R, Hermes M, Brunner B, Maichele A, Bocola M, Arand M, and Cronin A. Enhancing the enantioselectivity of an epoxide hydrolase by directed evolution. *Org Lett* 6: 177–180, 2004.
177. Richter MPO and Beck–Sickinger AG. Expressed protein ligation to obtain selectively modified aldo/keto reductases. *Prot Peptide Lett* 12: 777–781, 2005.
178. Rorabacher DB. Electron transfer by copper centers. *Chem Rev* 104: 651–697, 2004.
179. Rose K. Facile synthesis of homogeneous artificial proteins. *J Am Chem Soc* 116: 30–33, 1994.
180. Rosell A, Valencia E, Ochoa WF, Fita I, Pares X, and Farres J. Complete reversal of coenzyme specificity by concerted mutation of three consecutive residues in alcohol dehydrogenase. *J Biol Chem* 278: 40573–40580, 2003.
181. Rossmann MG, Moras D, and Olsen KW. Chemical and biological evolution of a nucleotide-binding protein. *Nature* 250: 194–199, 1974.
182. Rotello VM. Model systems for redox cofactor activity. *Curr Opin Chem Biol* 3: 747–751, 1999.
183. Rova U, Adrait A, Potsch S, Graslund A, and Thelander L. Evidence by mutagenesis that Tyr370 of the mouse ribonucleotide reductase R2 protein is the connecting link in the intersubunit radical transfer pathway. *J Biol Chem* 274: 23746–23751, 1999.
184. Roychowdhury–Saha M, Lato SM, Shank ED, and Burke DH. Flavin recognition by an RNA aptamer targeted toward FAD. *Biochemistry* 41: 2492–2499, 2002.
185. Rydberg P, Sigfridsson E, and Ryde U. On the role of the axial ligand in heme proteins: A theoretical study. *J Biol Inorg Chem* 9: 203–223, 2004.
186. Ryu Y and Schultz PG. Efficient incorporation of unnatural amino acids into proteins in *Escherichia coli*. *Nature Meth* 3: 263–265, 2006.
187. Sancho J. Flavodoxins: sequence, folding, binding, function and beyond. *Cell Mol Life Sci* 63: 855–864, 2006.
188. Satoh A, Kim JK, Miyahara I, Devreese B, Vandenberghe I, Hacisalihoglu A, Okajima T, Kuroda S, Adachi O, and Duine JA. Crystal structure of quinohemoprotein amine dehydrogenase from *Pseudomonas putida* Identification of a novel quinone cofactor encaged by multiple thioether cross-bridges. *J Biol Chem* 277: 2830–2834, 2002.
189. Saudan C and Johnsson K. Examining reactivity and specificity of cytochrome c peroxidase by using combinatorial mutagenesis. *Chembiochem* 3: 1097–1104, 2002.
190. Schäffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI, Koonin EV, and Altschul SF. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucl Acids Res* 29: 2994–3005, 2001.
191. Schlosrich J, Eley KL, Crowley PJ, and Bugg TD. Directed evolution of a non-heme-iron-dependent extradiol catechol dioxygenase: identification of mutants with intradiol oxidative cleavage activity. *Chembiochem* 7: 1899–1908, 2006.
192. Schmitzer AR, Lepine F, and Pelletier JN. Combinatorial exploration of the catalytic site of a drug-resistant dihydrofolate reductase: creating alternative functional configurations. Oxford U Press; *Prot Eng Design Selection* 17: 809–819. 2004.
193. Seyedsayamdost MR, Xie J, Chan CTY, Schultz PG, and Stubbe JA. Site-specific insertion of 3-aminotyrosine into subunit 2 of *E. coli* ribonucleotide reductase: Direct evidence for involvement of Y 730 and Y 731 in radical propagation. *J Am Chem Soc* 129: 15060–15071, 2007.
194. Seyedsayamdost MR, Yee CS, and Stubbe JA. Site-specific incorporation of fluorotyrosines into the R2 subunit of *E. coli* ribonucleotide reductase by expressed protein ligation. *Nature Protocols* 2: 1225–1235, 2007.
195. Seyedsayamdost MR, Yee CS, and Stubbe JA. Site-specific incorporation of fluorotyrosines into the R2 subunit of *E. coli* ribonucleotide reductase by expressed protein ligation. *Nature Protocols* 2: 1225–1235, 2007. Reference deleted at the galley proof stage.)
196. Sieber V, Martinez CA, and Arnold FH. Libraries of hybrid proteins from distantly related sequences. *Nature Biotech* 19: 456–460, 2001.
197. Skourtis SS and Beratan DN. High and low resolution of theories of protein electron transfer. *J Biol Inorg Chem* 2: 378–386, 1997.
198. Skourtis SS, Regan JJ, and Onuchic JN. Electron transfer in proteins: A novel approach for the description of donor-acceptor coupling. *J Phys Chem* 98: 3379–3388, 1994.
199. Stefan A, Radeghieri A, Gonzalezs Vara y Rodriguez A, and Hochkoeppler A. Directed evolution of b-galactosidase from *Escherichia coli* by mutator strains defective in the 3′ → 5′ exonuclease activity of DNA polymerase III. *FEBS Lett* 493: 139–143, 2001.
200. Stemmer WP. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* 370: 389–391, 1994.
201. Stone JR and Yang SP. Hydrogen peroxide: A signaling messenger. *Antioxid Redox Signal* 8: 243–270, 2006.
202. Stubbe J, Nocera DG, Yee CS, and Chang MCY. Radical initiation in the class I ribonucleotide reductase: Long-range proton-coupled electron transfer. *Chem Rev* 103: 2167–2201, 2003.
203. Stubbe JA. Protein radical involvement in biological catalysis? *Annu Rev Biochem* 58: 257–285, 1989.
204. Stubbe JA. Radicals with a controlled lifestyle. *Chem Commun* 2003: 2511–2513, 2003.
205. Stubbe JA, Seyedsayamdost M, Yee C, Chang M, and Nocera D. Long range radical initiaiton essential for class I ribonucleotide reductases: Direct evidence for amino acid radical intermediates. *FASEB J* 19: A1709, 2005.
206. Stuchebrukhov AA. Tunneling currents in electron transfer reactions in proteins. *J Chem Phys* 104: 8424–8432, 1996.

207. Stuchebrukhov AA. Long-distance electron tunneling in proteins. *Theoret Chem Acc* 110: 291–306, 2003.
208. Stuchebrukhov AA and Marcus RA. Theoretical study of electron transfer in ferrocytochromes. *J Phys Chem* 99: 7581–7590, 1995.
209. Suemori A and Iwakura M. A systematic and comprehensive combinatorial approach to simultaneously improve the activity, reaction specificity, and thermal stability of p-hydroxybenzoate hydroxylase. *J Biol Chem* 282: 19969–19978, 2007.
210. Tee KL and Schwaneberg U. Directed evolution of oxygenases: Screening systems, success stories and challenges. *Comb Chem High Troughput Screen* 10: 197–217, 2007.
211. Tobin MB, Gustafsson C, and Huisman GW. Directed evolution: The 'rational' basis for 'irrational' design. *Curr Opin Struct Biol* 10: 421–427, 2000.
212. Travascio P, Bennet AJ, Wang DY, and Sen D. A ribozyme and a catalytic DNA with peroxidase activity: Active sites versus cofactor-binding sites. *Chem Biol* 6: 779–787, 1999.
213. Tsukiji S, Pattnaik SB, and Suga H. An alcohol dehydrogenase ribozyme. *Nature Struct Biol* 10: 713–717, 2003.
214. Tsukiji S, Pattnaik SB, and Suga H. Reduction of an aldehyde by a NADH/Zn 2-dependent redox active ribozyme. *J Am Chem Soc* 126: 5044–5045, 2004.
215. Tu SC. Reduced flavin: donor and acceptor enzymes and mechanisms of channeling. *Antioxid Redox Signal* 3: 881–897, 2001.
216. Valderrama B, Ayala M, and Vazquez–Duhalt R. Suicide inactivaction of peroxidases and the challenge of engineering more robust enzymes. *Chem Biol* 9: 555–565, 2002.
217. Valderrama B, Garcia–Arellano H, Giansanti S, Baratto MC, Pogni R, and Vazquez–Duhalt R. Oxidative stabilization of iso-1-cytochrome c by redox-inspired protein engineering. *FASEB J* 20: E472–E481, 2006.
218. Valderrama B and Vazquez-Duhalt R. Electron-balance during the oxidative self-inactivation of cytochrome c. *J Mol Catal B: Enz* 35: 41–44, 2005.
219. van den Heuvel RH, Fraaije MW, Laane C, and van Berkel WJ. Enzymatic synthesis of vanillin. *J Agric Food Chem* 49: 2954–2958, 2001.
220. van den Heuvel RHH, van den Berg WAM, Rodavida S, and van Berkel WJH. Laboaratory-evolved vainillyl-alcohol oxidase produces natural vainillin. *J Biol Chem* 279: 33492–33500, 2004.
221. van der Donk WA and Zhao H. Recent developments in pyridine nucleotide regeneration. *Curr Opin Biotech* 14: 421–426, 2003.
222. van Montfort RL, Congreve M, Tisi D, Carr R, and Jhoti H. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* 423: 773–777, 2003.
223. Villegas JA, Mauk AG, and Vazquez–Duhalt R. A cytochrome c variant resistant to heme degradation by hydrogen peroxide. *Chem Biol* 7: 237–244, 2000.
224. Virnekas B, Ge L, Pluckthun A, Schneider KC, Wellnhofer G, and Moroney SE. Trinucleotide phosphoramidites: Ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucl Acids Res* 22: 5600–5607,
225. Vivancos AP, Castillo EA, Biteau B, Nicot C, Ayte J, Toledano MB, and Hidalgo E. A cysteine-sulfinic acid in peroxiredoxin regulates H2O2-sensing by the antioxidant Pap1 pathway. *Proc Natl Acad Sci USA* 102: 8875–8880, 2005.
226. Voigt CA, Martinez C, Wang ZG, Mayo SL, and Arnold FH. Protein building blocks preserved by recombination. *Nat Struct Biol* 9: 553–558, 2002.
227. Wallace CJA. *Protein Engineering by Semisynthesis*. Boca Raton, FLA: CRC Press LLC, 2008.
228. Wallace CJA, Mascagni P, Chait BT, Collawn JF, Paterson Y, Proudfoot AEI, and Kent SBH. Substitutions engineered by chemical synthesis at three conserved sites in mitochondrial cytochrome c. *J Biol Chem* 264: 15199–15209, 1989.
229. Wang K, Neumann H, Peak-Chew SY, and Chin JW. Evolved orthogonal ribosomes enhance the efficiency of synthetic genetic code expansion. *Nature Biotech* 25: 770–777, 2007.
230. Wang L and Schultz PG. A general approach for the generation of orthogonal tRNAs. *Chem Biol* 8: 883–890, 2004.
231. Wang L, Xie J, and Schultz PG. Expanding the genetic code. *Annu Rev Biophys Biomol Struct* 35: 225–249, 2006.
232. Wang SX, Mure M, Medzihradszky KF, Burlingame AL, Brown DE, Dooley DM, Smith AJ, Kagan HM, and Klinman JP. A crosslinked cofactor in lysyl oxidase: Redox function for amino acid side chains. *Science* 273: 1078, 1996.
233. Wengel J. Nucleic acid nanotechnology—towards Ångström-scale engineering. *Org Biomol Chem* 2: 277–280, 2004.
234. White HB. Coenzymes as fossils of an earlier metabolic state. *J Mol Evol* 7: 101–104, 1976.
235. Wierenga RK, Terpstra P, and Hol WG. Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. *J Mol Biol* 187: 101–107, 1986.
236. Wilson DS and Szostak JW. In vitro selection of functional nucleic acids. *Annu Rev Biochem* 68: 611–647, 1999.
237. Winkler JR. Electron tunneling pathways in proteins. *Curr Opin Chem Biol* 4: 192–198, 2000.
238. Woo HA, Chae HZ, Hwang SC, Yang KS, Kang SW, Kim K, and Rhee SG. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* 300: 653–656, 2003.
239. Xu MQ and Evans TC. Intein-mediated ligation and cyclization of expressed proteins. *Methods* 24: 257–277, 2001.
240. Yee CS, Chang MCY, Ge J, Nocera DG, and Stubbe J. 2, 3-Difluorotyrosine at position 356 of ribonucleotide reductase R2: A probe of long-range proton-coupled electron transfer. *J Am Chem Soc* 125: 506–510, 2003.
241. Zhang ZW, Alfonta L, Tian F, Bursulaya B, Uryu S, King DS, and Schultz PG. Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells. *Proc Natl Acad Sci USA* 101: 8882–8887, 2004.
242. Zhao H, Chockalingam K, and Chen Z. Directed evolution of enzymes and pathways for industrial biocatalysis. *Curr Opin Biotechnol* 13: 104–110, 2002.
243. Zhao H, Giver L, Shao Z, Affholter JA, and Arnold FH. Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat Biotechnol* 16: 258–261, 1998.
244. Zumarrága M, Bulter T, Shleev S, Polaina J, Martínez–Arias A, Plou FJ, Ballesteros A, and Alcalde M. *In vitro* evolution of a fungal laccase in high concentrations of organic cosolvents. *Chem Biol* 14: 1052–1064, 2007.

Address reprint requests to:
Brenda Valderrama
Departamento de Medicina Molecular y Bioprocesos
Instituto de Biotecnología
Universidad Nacional Autónoma de México
AP 510-3 Cuernavaca, Morelos 62250, México

E-mail: brenda@ibt.unam.mx

Date of first submission to ARS Central, April 18, 2008; date of final revised submission, August 14, 2008; date of acceptance, August 15, 2008.

This article has been cited by:

1. Kastoori Hingorani, Warwick Hillier. 2012. Perspectives for Photobiology in Molecular Solar Fuels. *Australian Journal of Chemistry* **65**:6, 643. [CrossRef]
2. Christopher C. Moser, J.L. Ross Anderson, P. Leslie Dutton. 2010. Guidelines for tunneling in enzymes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1797**:9, 1573-1586. [CrossRef]
3. Ángel T Martínez, Francisco J Ruiz-Dueñas, María Jesús Martínez, José C del Río, Ana Gutiérrez. 2009. Enzymatic delignification of plant cell wall: from nature to mill. *Current Opinion in Biotechnology* **20**:3, 348-357. [CrossRef]