Evolving enzymatic electrochemistry with rare or unnatural amino acids

Sahin, Selmihan; Milton, Ross Dean

How to cite

SAHIN, Selmihan, MILTON, Ross Dean. Evolving enzymatic electrochemistry with rare or unnatural amino acids. In: Current opinion in electrochemistry, 2022, vol. 35, p. 101102. doi: 10.1016/j.coelec.2022.101102

This publication URL: https://archive-ouverte.unige.ch/unige:173465
Publication DOI: 10.1016/j.coelec.2022.101102

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Electrochemistry

Review Article

Evolving enzymatic electrochemistry with rare or unnatural amino acids

Selmihan Sahin[1] and Ross D. Milton[1,2]

Abstract

Proper orientation of oxidoreductases on electrodes is important for efficient electron transfer in bioelectrochemical studies. Site-directed mutagenesis confers the ability to control the orientation of enzymes on electrodes in addition to modifying enzyme catalytic properties and understanding native electron transfer mechanisms and protein–protein interactions. Although numerous improvements have been achieved in the site-directed immobilization of enzymes, they are limited to the use of the 20 "standard" amino acids of the protein. This *opinion* considers the utilization of unnatural amino acids (UAAs) to introduce unique functional groups to proteins for their site-specific immobilization and subsequent enzymatic electrochemistry studies. Moreover, the importance of the site-specific incorporation of selenocysteine is described due to its potential to improve/alter the electrochemical properties.

Addresses

[1] University of Geneva, Department of Inorganic and Analytical Chemistry, Faculty of Sciences, Quai Ernest-Ansermet 30, 1211 Geneva 4, Switzerland

[2] National Centre of Competence in Research (NCCR) Catalysis, University of Geneva, Geneva, Switzerland

Corresponding authors: Milton, Ross D. (Ross.Milton@unige.ch); Sahin, Selmihan (Selmihan.Sahin@unige.ch)

Current Opinion in Electrochemistry 2022, **35**:101102

This review comes from a themed issue on **Emerging Opinions**

Edited by **Richard G. Compton**, **Hasuck Kim** and **Zhichuan J. Xu**

For complete overview about the section, refer Emerging Opinions

Available online 7 July 2022

https://doi.org/10.1016/j.coelec.2022.101102

Keywords

Selenocysteine, Unnatural amino acid, Site-specific mutation, Direct electron transfer, Site-directed immobilization.

Introduction

The field of enzymatic electrochemistry concerns itself with the use of enzymes as biocatalysts at electrode surfaces. Specifically, oxidoreductases (enzymes catalyzing the transfer of electrons between substrates) are enzymes of primary interest, where an electrode can replace one of an enzyme's substrates. This enables, in the case of electroenzymatic sensing, the generation of an electrocatalytic current in response to an enzyme's substrate and analyte of interest, such as glucose. Alternatively, renewable electricity can be supplied to an electrode-confined enzyme for the electroenzymatic synthesis of products of interest, such as molecular hydrogen (H_2). Both systems require the efficient transfer of electrons between oxidoreductases and electrodes, specifically with at least one of an enzyme's redox-active cofactors [1–3].

For decades, electron transfer between proteins, as well as between two or more cofactors within proteins, has been rationalized with interpretations of Marcus theory (Eq. (1)) [4].

$$k_{ET} = \frac{2\pi}{\hbar}\frac{H_{DA}^2}{\sqrt{4\pi\lambda RT}}e^{\frac{-(\Delta_r G^0+\lambda)^2}{4\lambda RT}} \quad (1)$$

In brief, semi-classical Marcus theory for non-adiabatic electron transfer relates the rate constant for electron transfer (k_{ET}) to (i) electronic coupling between donors/acceptors (H_{DA}), (ii) the Gibbs energy for the reaction ($\Delta_r G^0$, also known as the thermodynamic driving force), and (iii) the nuclear reorganization energies of the donor/acceptor [4,5]. Importantly, the matrix coupling Hamiltonian H_{DA} decays exponentially as a function of the distance (r_{DA}) and the medium (amino acids/solvent, termed an electron transfer decay parameter, β) between the donor and acceptor (Eq. (2)).

$$H_{DA}^2 = (H_{DA}^0)^2 e^{-\beta(r_{DA})} \quad (2)$$

Dutton and coworkers subsequently examined the X-ray crystal structures of 31 redox proteins alongside their experimentally determined values of k_{ET}, concluding that nature has primarily selected for proteins with redox cofactors <14 Å for robust, physiologically relevant electron transfer. In the case of enzyme/protein electrochemistry, this is often colloquially translated to "protein cofactors must be located <14 Å of an electrode surface." Two important points must be kept in mind: (i) the consideration of solely X-ray crystal structures discards any dynamic nature of proteins (and, thus,

variation(s) in k_{ET}) and (ii) electron transfer reactions do not immediately cease at distances >14 Å [6].

Keeping the importance of distance *vs.* k_{ET} in mind, many great efforts have been developed for enzymatic electrochemistry. First, mediated electron transfer (MET) employs diffusive redox-active species to shuttle electrons between electrodes and enzyme cofactors. Alternatively, these mediators can be covalently grafted to polymeric supports (yielding redox polymers) to both immobilize enzymes to electrode surfaces while also introducing a non-diffusive MET pathway. Second, much work has sought to control the orientation of enzymes on electrode surfaces for minimized electron transfer distances (and improved k_{ET}). This has primarily been achieved by modifying surfaces to be complementary to regions of charge or hydrophobicity/hydrophilicity on protein surfaces. Researchers have even designed electrode surfaces with functionalities to immobilize enzymes on electrode site selectively by using natural amino acids, such as lysine, tyrosine, and cysteine amino acids on protein surfaces. Among them, cysteine is arguably the most useful due to (i) its chemical reactivity, (ii) it being less abundant on protein surfaces than amino acids, such as lysine, and (iii) it can be also translocated or "mutated" to different positions on protein surfaces [7–9]. While attractive, this becomes moot in the case of proteins with multiple cysteines on their surfaces.

Herein, we review how unnatural amino acids (UAAs) with physiologically incompatible chemistries can be introduced to proteins to specifically improve electron transfer reactions. We then highlight the possible function of selenocysteine (Sec) mutation in the enzyme electrochemistry with the advance in the incorporation of Sec in enzymes by the genetic code expansion.

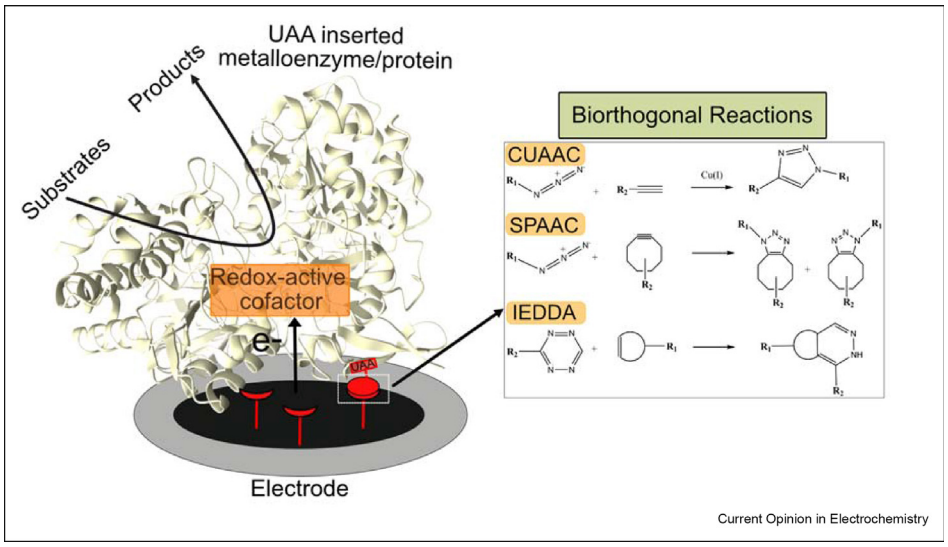
Unnatural amino acids for enzymatic electrochemistry

A more advanced approach that is attracting much attention is to introduce amino acid chemistries that go beyond the 20 "standard" proteinogenic amino acids. Specifically, researchers are increasingly turning toward the introduction of these UAAs to facilitate the specific anchoring of enzymes to electrodes or to introduce artificial electron-mediating amino acids. The "genetic code expansion" approach permits the incorporation of UAAs in the place of virtually any existing amino acids of a protein, providing a unique functional group that can be used to control enzyme orientation toward an electrode surface [10,11]. To achieve this, the "Amber codon suppression method" is mostly used, in which an orthogonal tRNA-aminoacyl tRNA synthetase pair is essential to decode an UAG amber stop codon for the incorporation of an UAA in the targeted position of the protein/enzyme [10,12]. Although the amber stop codon is used for the termination of mRNA translation in *Escherichia coli* (arguably the most relevant the enzyme electrochemists at this moment in time), it is possible to use modified *E. coli* strains that have had their endogenous amber codons recoded and relevant termination machinery removed (also known as amberless *E. coli*). This repurposes the amber codon (mutated in the gene sequence to a specific position) to have a single role: the specific insertion of a UAA [13–16].

UAAs with different functional groups, such as azides, alkynes, alkenes, and tetrazines, can be utilized for site-directed immobilization of enzymes/proteins on the functionalized surfaces through biorthogonal ligation with higher efficiency and specificity under mild conditions (pH 7, 37 °C) (Figure 1). The copper(I)-mediated cycloaddition (CuAAC) reaction, also known as the "click reaction," is a widely used biorthogonal reaction between azides and alkynes [17].

Amir et al. demonstrated the CuAAC reaction between para-azido-L-phenylalanine (AzF) incorporated within surface-displayed alcohol dehydrogenase II enzymes of living bacteria and gold electrodes functionalized with alkyne moieties [18]. Cyclic voltammograms of this UAA-containing enzyme and the wild-type enzyme (for non-specific immobilization), both of which were surface-displayed on *E. coli*, were analyzed, and the catalytic current for ethanol oxidation was found to increase approximately x10-fold. Their results also showed that there is an improvement of the heterogenous k_{ET} between alcohol dehydrogenase II and the electrode. Schlesinger et al. incorporated an alkyne-containing propargyl-L-lysine (PrK) residue in the blue copper oxidase enzyme CueO, which enabled its specific immobilization on glassy carbon electrodes functionalized with pyrene-diethyleneglycol-azide (PDAz) moieties by the CuAAC reaction (Figure 2a). Multistep amperometry was used to determine the heterogenous k_{ET} of the mutant and wild-type enzyme on the electrode. The authors demonstrated that the wild-type enzyme (non-specific orientation) adsorbed to the electrode with a mixed orientation. Importantly, the CueO containing PrK around the Cu I site showed better catalytic current (increased by up to ~8x-fold) and improved k_{ET} (9.92 s^{-1}) than the wild type (1.57 s^{-1}) [19]. Similarly, Algov et al. recently designed two different PrK-incorporated mutants of a flavin-adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) fused to a minimal cytochrome *c* domain (MCD) (yielding "FGM") for immobilization on electrodes functionalized with pyrene-diethyleneglycol-azide (PDAz) by CuAAC (Figure 2b). One of the mutants enables the enzyme to be covalently immobilized on the electrode close to its MCD, while the second enables covalent immobilization close to its FAD binding site. The authors also used a pyrene-carboxylic acid linker (PCA) for the non-specific immobilization of this FGM on electrodes used as a control. Remarkably, the mutant

Figure 1



Strategies for the site-directed immobilization of UAA-modified enzymes through biorthogonal reactions.

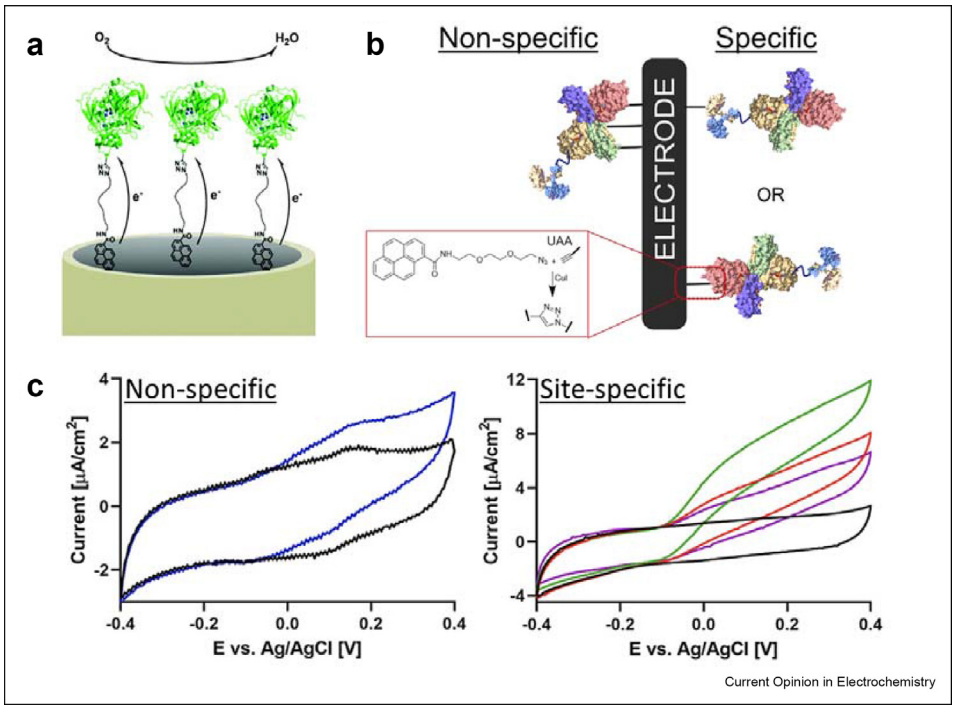
immobilized specifically at the MCD site demonstrated the largest k_{ET} (13.4 s^{-1}) and catalytic current (9.8 μA cm^{-2}), which were 2.5 and 10 times higher than that of the non-specifically oriented FGM, respectively (Figure 2c). Moreover, this permitted the authors to study the function of cofactors in the electron transfer mechanism of FGM in this study [20].

Since copper has the potential to inhibit enzyme activity, copper-free biorthogonal reactions (strain-promoted azido-alkyne cycloaddition (SPAAC) and inverse electron demand Diels–Alder (IEDDA)) have also been used as an alternative for the immobilization of enzymes/proteins on electrodes [21]. Guan et al. used the SPAAC reaction for site-specific immobilization of AzF containing-small laccase from *Streptomyces coelicolor* on electrodes modified with a cyclooctynyloxyethyl-1-pyrenebutyrate linker [22]. Ray et al. showed the immobilization of 3-amino-L-tyrosine-incorporated myoglobin on gold electrodes *via* an IEDDA reaction to form a homogeneous monolayer [23]. Although all these studies have shown improved k_{ET}, multiple challenges remain for the wider use of genetic code expansion for enzymatic electrochemistry, such as (i) low expression yields of unnatural enzymes, (ii) the cost of UAAs (when commercially available), and (iii) the need for 3D enzyme structural information to inform the positioning of UAAs [15,24]. In the case of the latter, homology-based modeling or computational structure predictions are expected to become increasingly employed and are expected to soon (in not already) negate this requirement [25,26].

One further use for UAAs in enzymatic electrochemistry (somewhat less relevant to non-catalytic protein electrochemistry) concerns the possibility of introducing redox-active amino acids, which serve to mimic typical diffusive electron mediators. In one example, Alfonta et al. demonstrated that the redox-active UAA 3,4-dihydroxy-L-phenylalanine (similar to *o*-hydroquinone electron mediators) could be incorporated within myoglobin [27]. Similarly, Yu et al. incorporated a different redox-active tyrosine analog (3-methoxy tyrosine) within myoglobin to introduce cytochrome *c* oxidase-like activity. Its lower $E^{0'}$ (179 mV more negative than tyrosine) resulted in an increase in myoglobin's oxidase activity, with the $2e^-$ reduction of O_2 to H_2O_2 being decreased by ~4x-fold and the $4e^-$ reduction of O_2 to H_2O being increased by ~5x-fold [28]. This could be envisaged as a strategy to produce enzymes containing redox mediators at the protein level, although it would presumably be necessary to introduce redox-mediating amino acids that provide sufficient driving force for the electron transfer reaction (much like the use of diffusive electron mediators). Thus, there would be no need to use toxic mediators in solution or within a redox polymer in electrochemical system to mediate ET between (metallo)enzymes and electrodes. However, it is very important to adjust the pK_a and $E^{0'}$ of redox-active amino acids depending on the target reaction. It is also important to note that the final $E^{0'}$ of redox-active UAAs could significantly differ once buried within a protein's secondary/tertiary structure, depending on effects such as solvation as well as contributions (electrostatics, H-bonding, *etc.*) from neighboring amino acids.

This would likely require the evolution of individual tRNA-aminoacyl tRNA synthetase pairs for each

Figure 2



(**a**) Schematic illustration of the site-directed immobilized CueO on glassy carbon electrodes, (**b**) schematic illustration of the expected orientation of non-specifically and site-directed immobilization of FGM on modified electrode surfaces, (**c**) CVs of non-specifically and site-specifically immobilized FGMs before (black) and after (red, green, purple, and blue; different colors represent different site-specific immobilization sites) the addition of glucose as the substrate (adapted with permission from Refs. [19,20]).

electron-mediating amino acid, which potentially impedes development in this area. In another study by Drienovská et al., a metal-chelating 2,2′-bipyridine-like UAA was incorporated to yield a Cu-chelating metalloenzyme [29]. This is exciting since different metals could potentially be chelated by this UAA (*i.e.*, Fe, thereby modulating E^0's), and the quaternization of the bipyridyl nitrogen groups would also yield electron mediators resembling viologens (and derivates thereof), which are electron mediators of interest to electrochemists studying enzymes such as hydrogenases [30]. This approach has the potential to improve k_{ET} between redox-active cofactors and electrodes by effectively shortening r_{DA} between individual redox-active centers *via* the UAA "stepping-stone," a principle employed in nature [6].

Exploiting amber codon suppression for the proteinogenic amino acid selenocysteine

Sec is known as the 21st amino acid genetically encoded in nature, where the sulfur of cysteine is replaced with a selenium atom. The *pK*a value of Sec is found to be 5.2 and lower than that of cysteine (8.5). While able to mimic cysteines by forming diselenide bonds in proteins [30], Sec is also attractive to enzymatic electrochemists due to its electrochemical and biocatalytic properties. Sec is observed to have a more negative reduction potential than cysteine ($E^{0'}_{Sec} = -388$ mV *vs.* SHE, $E^{0'}_{Cys} = -220$ mV *vs.* SHE), and its deprotonation at physiological pH results in greater nucleophilicity and improved reactions with electrophiles [32]. The majority of the known selenoproteins are oxidoreductases, where Sec plays a role in their catalytic activities. Importantly, the mutation of Sec residues to cysteines commonly results in the reduction of catalytic activities by 10−100-fold [32−34]. Moreover, Sec is found within certain hydrogenases and formate dehydrogenases, which are of interest to enzymatic electrochemists seeking to catalyze H_2 production or carbon dioxide (CO_2) reduction [35,36]. However, unlike mutagenesis of the 20 "standard" amino acids, Sec cannot be simply introduced by only the introduction of its corresponding opal (UGA) codon [32,34]. Although a review of Sec incorporation is beyond the scope of this opinion, it is important to mention that proteins containing Sec are obtained in low yield since Sec is encoded by another stop codon, UGA (opal), and employs a complex translation machinery that is in competition with this Sec-encoding stop codon. Importantly, however, the above-outlined amber codon suppression approach has been adapted to permit the site-specific insertion of Sec [31,37], which we believe

holds promise for oxidoreductase engineering in the domain of enzymatic electrochemistry. One recent study has demonstrated the replacement of four cysteine residues in a NiFe hydrogenase to Sec with amber codon suppression, where it was reported that Sec distorts the proton transfer pathway but improves the oxygen sensitivity of the enzyme [38]. In another study, a heme-coordinating cysteine residue in a cytochrome P450 was converted to its more nucleophilic Sec analog to determine the effect on enzymatic activity. The mutation resulted in a two-fold decrease in the catalytic activity, although substrate oxidation was augmented due to increased electron donation by the heme-cofactor [39]. We anticipate that the site-specific incorporation of Sec within enzymes of interest to the enzymatic electrochemistry community will be of great interest in the near future, due to its lower $E^{0'}$ as well as its central role in enzymes that reduce substrates such as H^+ and CO_2.

Conclusions and future perspectives

The incorporation of UAAs with non-natural chemical functionalities can introduce exciting biorthogonal handles to proteins, which is expected to benefit enzymatic electrochemistry. UAA incorporation has been exploited for different aims in biological applications [10]. Regarding enzymatic electrochemistry, this method can (i) help one study enzyme mechanisms, (ii) introduce new functionalities, and (iii) also improve catalytic properties and stability of enzymes, which have possibility to contribute to the efficiency, sensitivity, and stability of enzymes during electrochemical studies and biotechnological application. We anticipate that, with the development and commercialization of new recombinant protein production methods, UAAs will become increasingly attractive to enzymatic electrochemists seeking to optimize heterogeneous electron transfer. Expanding on the outlined approaches of UAA insertion, we anticipate that the use of amber codon suppression for the relatively simplified site-specific insertion of Sec will also be of importance to researchers studying electroenzymatic H_2 production and CO_2 reduction, as well as more generally to intraprotein electron transfer. However, significant limitations remain that should be considered for the application of this technology for enzymatic electrochemistry. The low activity and specificity of the orthogonal translation machinery for UAAs (especially when the structure of a UAA is significantly changed) can cause protein truncation (because of the competing UAG stop codon) and low UAA incorporation efficiencies, even if RF1-depleted (amberless) bacteria is used [40,41]. Because of this problem, low activities of mutant enzymes can be obtained even if the protein yield is comparable (misincorporated proteins), which also affects the specificity of the electrochemical reactions. Further improvement of the UAA incorporation efficiency and fidelity is also possible with the engineering of orthogonal translation systems and ribosomes and will enable an increasing number of studies for electrochemistry research. Another limitation is the lack of advanced high-resolution techniques (such as spectroscopic and microscopic methods) to provide insight into protein–electrode interactions, important for demonstrating the orientation, loading, and activities of immobilized enzymes. Continued advances on these fronts will aid the use of oxidoreductases and accelerate the use of UAA- and Sec-inserted proteins in electrochemical applications.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
Ross MILTON reports financial support was provided by Swiss National Science Foundation. Selmihan SAHIN reports was provided by European Commission Marie Sklodowska-Curie Actions.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 101024443. This publication was created as part of NCCR Catalysis (grant number 180544), a National Centre of Competence in Research funded by the Swiss National Science Foundation. RDM thanks the Swiss National Science Foundation for support (grant number 200021_191985).

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Hitaishi VP, Clement R, Bourassin N, Baaden M, de Poulpiquet A, Sacquin-Mora S, Ciaccafava A, Lojou E: **Controlling redox enzyme orientation at planar electrodes**. *Catalysts* 2018, **8**, https://doi.org/10.3390/catal8050192.

2. Flexer V, Brun N: *Fundamentals of enzymatic electrochemical systems*. 2017:3–50, https://doi.org/10.1142/9781786343543_0001.

3. Algov I, Grushka J, Zarivach R, Alfonta L: **Highly efficient flavin-adenine dinucleotide glucose dehydrogenase fused to a minimal cytochrome C domain**. *J Am Chem Soc* 2017, **139**: 17217–17220, https://doi.org/10.1021/jacs.7b07011.

4. ** Marcus RA, Sutin N: **Electron transfers in chemistry and biology**. *Biochim Biophys Acta* 1985, **811**:265–322, https://doi.org/10.1016/0304-4173(85)90014-X.
This comprehensive work outlines classical and semi-classical electron transfer theories, in inorganic systems as well as in biological electron transfer systems.

5. ** Jeuken LJC: **Conformational reorganisation in interfacial protein electron transfer**. *Biochim Biophys Acta Bioenerg* 2003, **1604**:67–76, https://doi.org/10.1016/S0005-2728(03)00026-4.
This review considers semi-classical Marcus theory in relation to protein electrochemistry, discussing how protein electrochemistry can be observed to deviate from Marcus theory.

6. ** Page CC, Moser CC, Chen X, Dutton PL: **Natural engineering principles of electron tunnelling in biological oxidation-reduction**. *Nature* 1999, **402**:47–52, https://doi.org/10.1038/46972.
This work compares experimental electron transfer rates in proteins with their X-ray crystal structures, leading to an expression that permits

the reader to predict protein electron transfer rate constants with knowledge of parameters such as (i) driving force ($\Delta_r G^0$), electron donor–acceptor distance (r), and the packing of amino acids bewteen a donot and acceptor.

7. Sévery L, Szczerbiński J, Taskin M, Tuncay I, Brandalise Nunes F, Cignarella C, Tocci G, Blacque O, Osterwalder J, Zenobi R, Iannuzzi M, Tilley SD: **Immobilization of molecular catalysts on electrode surfaces using host–guest interactions**. *Nat Chem* 2021, **13**:523–529, https://doi.org/10.1038/s41557-021-00652-y.

8. * Meneghello M, Al-Lolage FA, Ma S, Ludwig R, Bartlett PN: **Studying direct electron transfer by site-directed immobilization of cellobiose dehydrogenase**. *Chemelectrochem* 2019, **6**:700–713, https://doi.org/10.1002/celc.201801503.

This article demonstrates the use of repositioned individual cysteine residues for thiol-maleimide Michael addition reactions with electrodes, ultimately uncovering the heterogeneous electron transfer pathway between this enzyme and electrode surfaces.

9. Al-Lolage FA, Meneghello M, Ma S, Ludwig R, Bartlett PN: **A flexible method for the stable, covalent immobilization of enzymes at electrode surfaces**. *Chemelectrochem* 2017, **4**: 1528–1534, https://doi.org/10.1002/celc.201700135.

10. Pagar AD, Patil MD, Flood DT, Yoo TH, Dawson PE, Yun H: **Recent advances in biocatalysis with chemical modification and expanded amino acid alphabet**. *Chem Rev* 2021, **121**: 6173–6245, https://doi.org/10.1021/acs.chemrev.0c01201.

11. Wang T, Liang C, Xu H, An Y, Xiao S, Zheng M, Liu L, Nie L: **Incorporation of nonstandard amino acids into proteins: principles and applications**. *World J Microbiol Biotechnol* 2020, **36**:2918–2924, https://doi.org/10.1007/s11274-020-02837-y.

12. Naowarojna N, Cheng R, Lopez J, Wong C, Qiao L, Liu P: **Chemical modifications of proteins and their applications in metalloenzyme studies**. *Synth. Syst. Biotechnol.* 2021, **6**:32–49, https://doi.org/10.1016/j.synbio.2021.01.001.

13. Lajoie M, Rovner A, Goodman D, Aerni H, Haimovich A, Kuznetsov G, Mercer J, Wang H, Carr P, Mosberg J, Rohland N, Schultz P, Jacobson J, Rinehart J, Church G, Isaacs F: **Genomically recoded organisms expand biological functions**. *Science* 2013, **342**:357–360, https://doi.org/10.1126/science.1242902.

14. Santoro SW, Wang L, Herberich B, King DS, Schultz PG: **An efficient system for the evolution of aminoacyl-tRNA synthetase specificity**. *Nat Biotechnol* 2002, **20**:1044–1048, https://doi.org/10.1038/nbt742.

15. Smolskaya S, Andreev YA: **Site-specific incorporation of unnatural amino acids into Escherichia coli recombinant protein: methodology development and recent achievement**. *Biomolecules* 2019, **9**:255–272, https://doi.org/10.3390/biom9070255.

16. Chatterjee A, Sun SB, Furman JL, Xiao H, Schultz PG: **A versatile platform for single- and multiple-unnatural amino acid mutagenesis in escherichia coli**. *Biochemistry* 2013, **52**: 1828–1837, https://doi.org/10.1021/bi4000244.

17. Maza JC, Nimmo ZM, Young DD: **Expanding the scope of alkyne-mediated bioconjugations utilizing unnatural amino acids**. *Chem Commun* 2016, **52**:88–91, https://doi.org/10.1039/c5cc08287k.

18. Amir L, Carnally SA, Rayo J, Rosenne S, Melamed Yerushalmi S, Schlesinger O, Meijler MM, Alfonta L: **Surface display of a redox enzyme and its site-specific wiring to gold electrodes**. *J Am Chem Soc* 2013, **135**:70–73, https://doi.org/10.1021/ja310556n.

19. ** Schlesinger O, Pasi M, Dandela R, Meijler MM, Alfonta L: **Electron transfer rate analysis of a site-specifically wired copper oxidase**. *Phys Chem Chem Phys* 2018, **20**:6159–6166, https://doi.org/10.1039/c8cp00041g.

This key study demonstrates the tailoring of the metalloenzyme CueO on electrode surfaces by the incorporation of a UAA. The effect of site-directed immobilization on the DET charateristics of the enzyme is provided.

20. ** Algov I, Feiertag A, Alfonta L: **Site-specifically wired and oriented glucose dehydrogenase fused to a minimal cytochrome with high glucose sensing sensitivity**. *Biosens Bioelectron* 2021, **180**, https://doi.org/10.1016/j.bios.2021.113117.

This outstanding paper describes how the orientation of glucose dehydrogenase on electrode surfaces affects the activity, stability and electron transfer. Importantly, a minimal cytochrome unit was fused to glucose dehydrogenase to yield an artificial electron transfer mechanism that also exploited the site-specifically insertion of UAAs.

21. Kim E, Koo H: **Biomedical applications of copper-free click chemistry:: in vitro, in vivo, and ex vivo**. *Chem Sci* 2019, **10**: 7835–7851, https://doi.org/10.1039/c9sc03368h.

22. Guan D, Kurra Y, Liu W, Chen Z: **A click chemistry approach to site-specific immobilization of a small laccase enables efficient direct electron transfer in a biocathode**. *Chem Commun* 2015, **51**:2522–2525, https://doi.org/10.1039/c4cc09179e.

23. Ray S, Chand S, Zhang Y, Nussbaum S, Rajeshwar K, Perera R: **Implications of active site orientation in myoglobin for direct electron transfer and electrocatalysis based on monolayer and multilayer covalent immobilization on gold electrodes**. *Electrochim Acta* 2013, **99**:85–93, https://doi.org/10.1016/j.electacta.2013.03.080.

24. ** Algov I, Alfonta L: **Use of protein engineering to elucidate electron transfer pathways between proteins and electrodes**. *ACS Meas. Sci. Au.* 2022, **2**:78–90, https://doi.org/10.1021/acsmeasuresciau.1c00038.

This review summarizes protein engineering strategies for optimal electron transfer between electrodes and proteins. Strategies including protein truncation and deletion, point mutations, protein fusion and UAA incorporation are presented with detailed examples.

25. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, De Beer TAP, Rempfer C, Bordoli L, Lepore R, Schwede T: **SWISS-MODEL: homology modelling of protein structures and complexes**. *Nucleic Acids Res* 2018, **46**: W296–W303, https://doi.org/10.1093/nar/gky427.

26. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D: **Highly accurate protein structure prediction with AlphaFold**. *Nature* 2021, **596**:583–589, https://doi.org/10.1038/s41586-021-03819-2.

27. Alfonta L, Zhang Z, Uryu S, Loo JA, Schultz PG: **Site-specific incorporation of a redox-active amino acid into proteins**. *J Am Chem Soc* 2003, **125**:14662–14663, https://doi.org/10.1021/ja038242x.

28. Yu Y, Zhou Q, Wang L, Liu X, Zhang W, Hu M, Dong J, Li J, Lv X, Ouyang H, Li H, Gao F, Gong W, Lu Y, Wang J: **Significant improvement of oxidase activity through the generic incorporation of a redox-active unnatural amino acid**. *Chem Sci* 2015, **6**:3881–3885, https://doi.org/10.1039/C5SC01126D.

29. Drienovská I, Rioz-Martínez A, Draksharapu A, Roelfes G: **Novel artificial metalloenzymes by *in vivo* incorporation of metal-binding unnatural amino acids**. *Chem Sci* 2015, **6**:770–776, https://doi.org/10.1039/C4SC01525H.

30. Hardt S, Stapf S, Filmon DT, Birrell JA, Rüdiger O, Fourmond V, Léger C, Plumeré N: **Reversible H2 oxidation and evolution by hydrogenase embedded in a redox polymer film**. *Nat. Catal.* 2021, **4**:251–258, https://doi.org/10.1038/s41929-021-00586-1.

31. * Thyer R, Shroff R, Klein DR, D'Oelsnitz S, Cotham VC, Byrom M, Brodbelt JS, Ellington AD: **Custom selenoprotein production enabled by laboratory evolution of recoded bacterial strains**. *Nat Biotechnol* 2018, **36**:624–631, https://doi.org/10.1038/nbt.4154.

A significantly advanced and innovative strategy for the highly efficient incorporation of selenocysteine with an engineered *E. coli* strain is presented.

32. Mousa R, Notis Dardashti R, Metanis N: **Selenium and selenocysteine in protein chemistry**. *Angew Chem, Int Ed* 2017, **56**: 15818–15827, https://doi.org/10.1002/anie.201706876.

33. Wang Y, Liu P, Chang J, Xu Y, Wang J: **Site-specific selenocysteine incorporation into proteins by genetic engineering**. *Chembiochem* 2021, **22**:2918–2924, https://doi.org/10.1002/cbic.202100124.

34. Arnér ESJ: **Selenoproteins-What unique properties can arise with selenocysteine in place of cysteine?** *Exp Cell Res* 2010, **316**:1296–1303, https://doi.org/10.1016/j.yexcr.2010.02.032.

35. Boyington JC, Gladyshev V, V Khangulov S, Stadtman TC, Sun PD: **Crystal structure of formate dehydrogenase H: catalysis crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe involving Mo, molybdopterin, selenocysteine, and an Fe_4S_4 cluster**. *Science* 1997, **275**:1305–1308, https://doi.org/10.1126/science.275.5304.1305.

36. Marques MC, Tapia C, Gutiérrez-Sanz O, Ramos AR, Keller KL, Wall JD, De Lacey AL, Matias PM, Pereira IAC: **The direct role of selenocysteine in [NiFeSe] hydrogenase maturation and catalysis**. *Nat Chem Biol* 2017, **13**:544–550, https://doi.org/10.1038/nchembio.2335.

37. Cheng Q, Arnér ESJ: **Selenocysteine insertion at a predefined UAG codon in a release factor 1 (RF1)-depleted Escherichia coli host strain bypasses species barriers in recombinant selenoprotein translation**. *J Biol Chem* 2017, **292**:5476–5487, https://doi.org/10.1074/jbc.M117.776310.

38. ** Evans RM, Krahn N, Murphy BJ, Lee H, Armstrong FA, Söll D: **Selective cysteine-to-selenocysteine changes in a [NiFe]-hydrogenase confirm a special position for catalysis and oxygen tolerance**. *Proc Natl Acad Sci USA* 2021, **118**, e2100921118, https://doi.org/10.1073/pnas.2100921118.
An innovative approach that employs amber codon supression to selectively insert selenocysteine mutations to investigate its functional role in the metalloenzyme [NiFe(Se)]-hydrogenase. The authors describe the changes in kinetic and electrochemical properties of the enzyme in detail, as a function of selenocysteine insertion.

39. Aldag C, Gromov IA, García-Rubio I, von Koenig K, Schlichting I, Jaun B, Hilvert D: **Probing the role of the proximal heme ligand in cytochrome P450cam by recombinant incorporation of selenocysteine**. *Proc Natl Acad Sci USA* 2009, **106**:5481–5486. https://www.pnas.org/doi/10.1073/pnas.0810503106.

40. Shandell MA, Tan Z, Cornish VW: **Genetic code expansion: a brief history and perspective**. *Biochemistry* 2021, **60**: 3455–3469, https://doi.org/10.1021/acs.biochem.1c00286.

41. Zhao H, Ding W, Zang J, Yang Y, Liu C, Hu L, Chen Y, Liu G, Fang Y, Yuan Y, Lin S: **Directed-evolution of translation system for efficient unnatural amino acids incorporation and generalizable synthetic auxotroph construction**. *Nat Commun* 2021, **12**:1–12, https://doi.org/10.1038/s41467-021-27399-x. Article: 7039.