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Molecular "Wiring" of Plasma Amine Oxidase: Green and Enzyme Friendly Approaches

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Abstract:

The study describes two approaches to enhance oxidoreductases. Both target molecular "wiring" of enzymes using green processes. The concepts were tested on plasma amine oxidase (PAO). In the first procedure PAO was transiently exposed to an ionic liquid (IL) in the presence of redox molecules, which resulted in partial unfolding. During subsequent dialysis, the enzyme refolded entrapping redox units and affording shorter distances for electron tunneling, hence a molecular "wire" to PAO's prosthetic groups. The other procedure described herein was totally reagentless, using high hydraulic pressure (HHP) to partially denature PAO (in the presence of redox molecules) followed by dialysis and refolding. The twoh approaches to enzyme "wiring" are discussed comparatively from the point of view of the parameters used during the procedure, residual enzyme activity, nature of the modifier, interaction between PAO and the redox molecules, and stability over time. The most active modified PAO (PAO-ME) from each series was tested in a biosensor for amine detection, towards applications in the food industry and clinical laboratory. Our approaches used "green" reagents (IL) and were made enzyme-friendly as well by the choice of "wires".

Keywords: protein engineering**,** enzyme "wiring", biosensors

1. Introduction

Redox enzymes facilitate electron transfer in diverse processes, from respiration, to photosynthesis, from apoptosis to nitrogen fixation, to biosynthetic reactions. Recently, their applications in implantable and non-invasive medical devices and biofuel cells made the object of many publications as did amperometric biosensors in which redox enzymes were integrated in different types of electrodes [1].

Plasma amine oxidase (PAO) is a copper enzyme which catalyzes the oxidation of primary amines to their corresponding aldehydes, as illustrated in equation 1.

$$RCH_2NH_3^+ + H_2O + O_2 \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1)$$

PAO is a homodimeric enzyme containing as cofactors one copper II ion and one redox-active organic molecule, 2,4,5-trihydroxyphenylalaninequinone (topaquinone, TPQ) covalently attached-in each subunit (Fig. 1) [2, 3]. The TPQ cofactor is known to contribute in the reductive half-reaction by creating a covalent adduct with the amine substrate. This Schiff base complex is further deprotonated by an active-site basic group leading to the formation of the reduced cofactor. The subsequent hydrolysis of the Schiff base results in the release of the product aldehyde and the aminoresorcinol form of the reduced cofactor (TPQred). In the oxidative half-reaction, TPQred is reoxidized by dioxygen to the initial oxidized state (TPQox) and ammonia is released. The role of the enzyme-bound copper ion is suggested as essential (the electron shuttle) for the oxidative half-reaction. PAO has an important biological role in detoxifying xenobiotic amines, in cell-cell recognition, and the hydrogen peroxide released in the oxidative deamination helps in cell wall maturation and lignification as well as in self-wound healing. Among its applications, some biosensors for clinical laboratory and for the food industry have been reported [4, 5] and are an area of high interest.

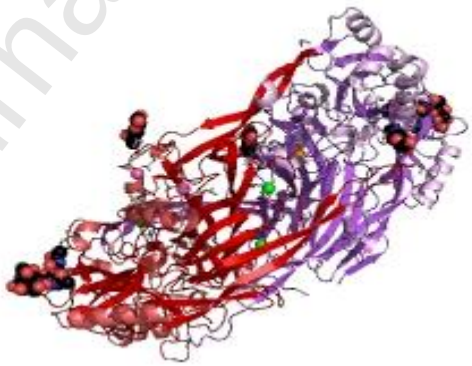

Figure 1. Crystal structure of copper-containing plasma amine oxidase

Efficient electron exchange between oxidoreductases and other redox species or electrodes depends on proximity and orientation between donor and acceptor redox centers. Gray and coworkers demonstrated that electron transfer in proteins is a tunneling process in which distance has an important role [6]. This means that, due to the insulating effect of the protein layers, only those prosthetic groups which are close to the protein surface will be actually useful

/ involved in electron transfer [7]. One solution to this problem was to immobilize enzymes on electrodes together with mediators [8-10]. This procedure is a labor-intensive one and it typically results in important loss of enzyme activity [11] and mediator leakage [12].

To address these drawbacks, Adam Heller and his co-workers included glucose oxidase (GOX) into a conductive gel which was deposited onto an electrode and coined the term "wiring" enzymes. The result was the decrease in the electron tunneling distance between the redox centers inside the enzyme and the electrode hence a more efficient electron transfer. For a while, such a device which artificially connects the external electrode transducer to an enzyme (a peroxidase) redox centers was even available commercially [13].

Using the same enzyme model, Calvo *et al* reported self-assembled nanostructures comprised of enzyme layers alternating with osmium derivatized poly(allylamine) layers, acting as redox relays [14]. They reported that the "wiring efficiency" (equated to the rate of the oxidation of the reduced coenzyme, flavin adenine dinucleotide, $FADH_2$) was controlled by the diffusion of electrons across layers. Another approach to enzyme "wiring" used the prosthetic group of an enzyme as "wire" and reported sizable enhancement of the electron transfer kinetics [15]. The enhancement in activity afforded was dependent on the status of the prosthetic group within the 3-D structure of the enzyme. Yet another strategy to establish an electrically conductive connection between an enzyme's active sites and an electrode was reported in [16]. Mehmeti *et al.* separated the coenzyme (FAD) from a redox enzyme, covalently bound it to an electron shuttle (graphene nanoribbons), and recombined the apoenzyme with the coenzyme-shuttle complex (based on affinity). Applications targeting biofuel cells (BFC) fabrication report "wiring" of redox enzymes by immobilization in nanocavities of mesoporous structures used to make electrodes for the cells, resulting in enhanced mass and electron transfer hence BFC with higher performance [1].

While "wiring" of enzymes was praised by some as a good replacement of mediated electron transfer (MET) with its shortcomings and said to represent a direct electron transfer (DET) alternative, a reagentless one [17], the difference between MET/DET is not very clear in some of the reported sytems [14, 16, 17]. In all these cases, while the enzyme "wiring" principle is observed (decreasing the distance the electrons need to travel during transfer), the "wire" is an external one, actually mediating the electron transfer between enzyme active sites and external redox center.

The present paper proposes a molecular approach to enzyme "wiring". To this end, two different procedures were tested using PAO as model enzyme. In both the target was not to make the bulk of the protein conductive but to establish a preferential path for electron transfer (direct electron transfer, DET). To this end PAO was transiently exposed to an ionic liquid (IL) in the presence of redox molecules, which resulted in partial unfolding. Following subsequent dialysis, the enzyme refolded entrapping redox units present. Shorter distances for the electrons to tunnel over and a molecular "wire" to PAO's prosthetic groups were the result. The other procedure described herein was totally reagentless, using high hydraulic pressure (HHP) to partially denature PAO (in the presence of redox molecules) followed by dialysis and refolding. Our approaches used "green" reagents (IL) and were made enzyme-friendly as well by the choice of "wires".

2. Materials and Methods

2.1 Materials

Plasma amine oxidase (amine:oxigenoxidoreductase, deaminating, EC 1.4.3.21) was purchased from Worthington Biochemical Corporation. 1-Ethyl-3-methylimidazolium tetrafluoroborate (emimBF_4, Fig. 2a), flavin adenine dinucleotide (FAD, Fig. 2b), benzylamine (Fig. 2c), ferrous sulfate, pyridoxal phosphate (PLP, Fig. 2d), lipoic acid (LA, Fig. 2e), and copper (II) sulfate (CU) were bought from Sigma-Aldrich and used without further purification. All solutions were made in deionized water.

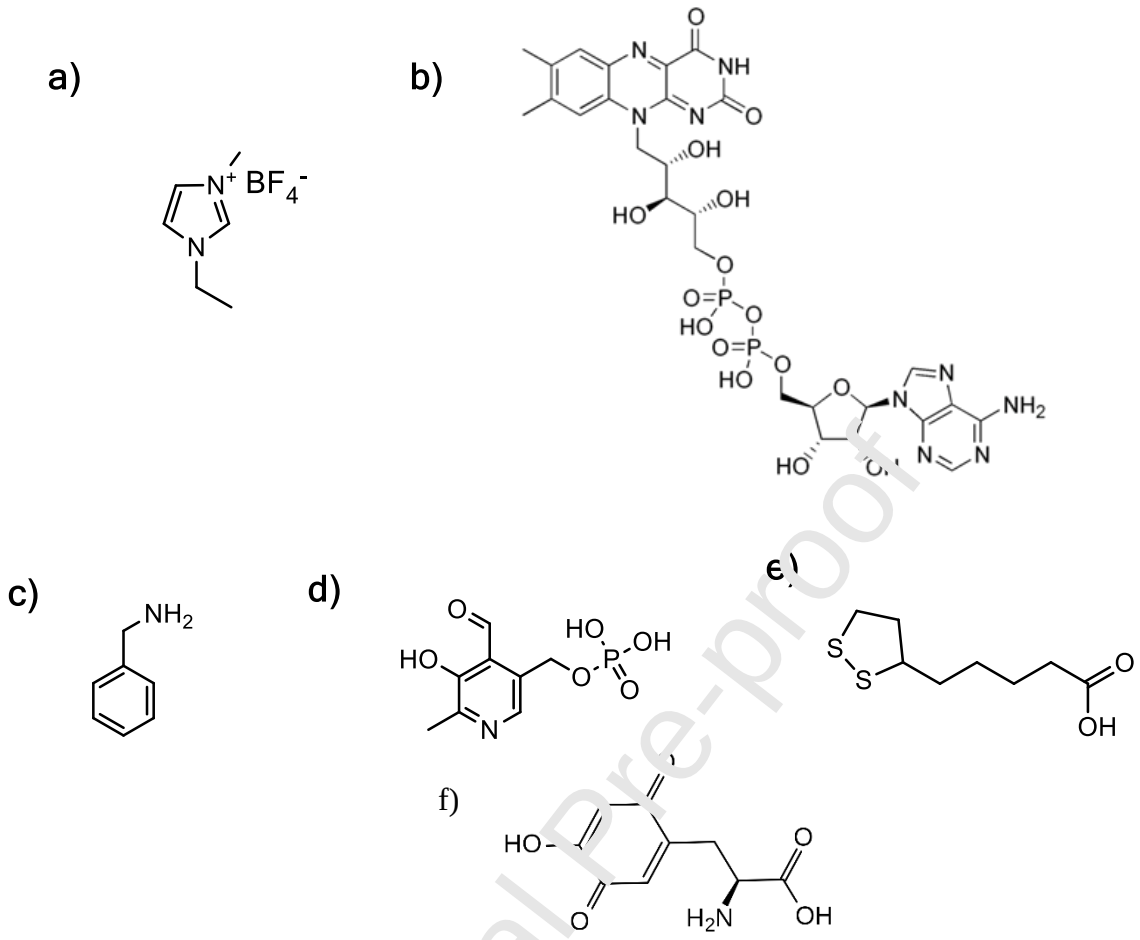

Figure 2. (a) 1-Ethyl-3-methylimidazolium tetrafluoroborate (emimBF_4); (b) flavin adenine dinucleotide (FAD); (c) benzylamine; (d) pyridoxal phosphate (PLP); (e) □–lipoic acid (LA), (f) topaquinone (TPQ)

2.2 Methods

2.2.1. PAO "wiring" using emimBF_4

Eight enzyme samples (containing 600 Tabor units, TU, of PAO each) were used for the modification processes using emimBF_4, in parallel procedures. Table 1 shows the composition of each preparative mixture. Each mixture contained 2 mM modifier and 0.75 mL deionized water added.

Table 1 Composition of the mixtures used for PAO "wiring" using emimBF_4

Sample*	PAO (mg)	FAD (mg)	PLP (mg)	$CuSO_4$ (mg)	LA (mg)	IL (mL)	Water (mL)
IL-FAD	20.21	1.37	-	-	-	0.25	0.75
IL-PLP	21.22	-	0.494	-	-	0.25	0.75

IL-CU	20.32	-	-	0.498	-	0.25	0.75
IL-LA	21.02	-	-	-	0.413	0.25	0.75
IL-FAD/CU	22.01	1.37	-	0.498	-	0.25	0.75
IL-FAD/LA	20.64	1.37	-	-	0.413	0.25	0.75
IL-PLP/CU	20.85	-	0.494	0.498	-	0.25	0.75
IL-PLP/LA	21.43	-	0.494	-	0.413	0.25	0.75

*IL – modifier 1/modifier 2 signifies modification in the presence of IL and one or two modifying species

The mixtures were kept under magnetic stirring for 2 hours, at 24°C. Dialysis followed (for 24 hours) under gentle magnetic stirring, against 67 mM phosphate buffer pH 7.2 and using a dialysis membrane with a cutoff molecular weight of 3kDa. After dialysis, the renatured/modified enzyme (PAO-ME) samples were lyophilized and then stored at -20°C. The buffer from dialysis was saved to analyze its modifier(s) content.

2.2.2. PAO "wired" using high hydraulic pressure

The ten synthetic mixtures used in the HHP modification are presented in Table 2. The compression times shown in the last column reflect the duration each PAO sample was subjected to high hydraulic pressure (HHP), with or without a modifier present (at 2mM). A Pressure Biosciences Inc. (PBI) High Pressure Generator was used to press samples, under 325MPa, for 60 minutes while others were compressed at 325MPa for 30 minutes. Each sample, after exposure to high pressure, underwent dialysis for 24 hours against phosphate buffer 67 mM pH 7.2 and subsequent lyophilization as in 2.2.1. They were stored at -20°C. The buffer from dialysis was saved to analyze its modifier content.

Table 2: Composition of the mixtures used in the "wiring" at high hydraulic pressure

Sample*	PAO (mg)	FAD (mg)	PLP (mg)	$CuSO_4$ (mg)	LA (mg)	Buffer (mL)	Time (min)
HP60[a]	23.20	-	-	-	-	0.5	60
HP60-FAD[b]	21.60	1.14	-	-	-	0.5	60
HP60-PLP[b]	21.40	-	0.41	-	-	0.5	60
HP60-CU[b]	24.00	-		0.42		0.5	60
HP60-LA[b]	22.50	-	-	-	0.34	0.5	60
HP30[a]	21.61	-	-	-	-	0.5	30
HP30-FAD[b]	23.80	1.14	-	-	-	0.5	30

HP30-PLP [b]	21.30	-	0.41	-	-	0.5	30
HP30-CU	21.30	-	-	0.42	-	0.5	30
HP30-LA	23.60	-	-	-	0.34	0.5	30

[a] HP60 is PAO exposed to 325 MPa for 60 min; HP30 is PAO exposed to 325 MPa for 30 min; [b]HP60-modifier is PAO exposed to 325 MPa for 60 min in the presence of a modifier (as shown); HP30-modifier is PAO exposed to 325 MPa for 30 min in the presence of a modifier (as shown).

2.2.3. Analysis of the modifier entrapped in PAO-ME

The modifier uptakes for each PAO-ME from 2.2.1. and 2.2.2. were estimated indirectly using a spectrophotometric measurement of each modifier species (Table 3) in the wash from the dialysis procedures. The concentration of the modifier in the wash was calculated using Beer's Law and the amount entrapped in the refolded enzyme was calculated by subtraction from the amount used in the synthetic mixture.

Table 3 Spectrophotometric data for the modifiers

Modifiers	Absorbance Wavelength (nm)	Extinction Coefficient ($M^{-1}cm^{-1}$)
PLP	388	5020
FAD	375	9400
LA	390	660
$CuSO_4,5H_2O$	600	942

2.2.4. PAO Activity Assay

The assay used is that of Tabor *et al.* [18, 19] modified by Worthington [20, 21]. The reaction velocity is measured as increase in absorbance at 250 nm due to peroxide formation (Eq. 2). One International Unit, defined as the oxidation of one micromole of benzylamine/minute at 25°C and pH 7.2, is equivalent to 4330 Tabor units, customary used when activity of PAO is measured [20]. A solution of benzylamine in 67 mM potassium phosphate buffer pH 7.2 was used as substrate. For the assays, 10 mg lyophilized powder (PAO and the modified enzyme, PAO-ME, respectively) were dissolved in 1 mL 67 mM potassium phosphate buffer, pH 7.2. Measurements were done in a Perkin-Elmer Lambda-35 UV-Vis spectrophotometer, using Winlab software, at 250 nm and 25°C. Quarz cuvettes containing 2.8 mL of buffer and 0.1 mL

benzylamine solution were incubated for 5 minutes at 25°C. Subsequently, 0.1 mL of enzyme solution were added and the increase in absorbance at 250 nm was recorded for 6 minutes. ΔA250/min was calculated from the linear portion of the curve, in TU and in International Units (IU) (Eq.2). A 2 min lag was observed after which the absorbance increase was linear to ~0.75).

$$\text{Activity (IU)} = \text{Activity (Tabor Unit)} \times \frac{1\ \text{International Unit}}{4330\ \text{Tabor Unit}} = \frac{\text{Slope} \times 1000}{13.0 \times \frac{10\ \text{mg enzyme}}{\text{ml reaction mixture}}} \times \frac{1\ \text{International Unit}}{4330\ \text{Tabor Unit}} \quad (2)$$

2.2.5. Lyophilization of PAO-ME samples

Lyophilization of the PAO-ME samples was done using a Labconco FreeZone 2.5 Freeze Dry System (at -55^{o}C and 0.05 mbar). This procedure carries the potential risk of decrease in activity of the enzyme or even denaturation. To account for this, a sample of the native PAO was assayed before and after lyophilization. The results in Table 4 show an approximate 70% loss in activity following freeze drying.

Table 4: Effect of lyophilization on PAO activity

PAO	Activity (TU)	Activity (IU)	Residual Activity (%Native)
0[a]	0.2179	$5.033*10^{-5}$	-
XX[b]	0.06685	$1.544*10^{-5}$	30.68

[a] 0 – Native PAO; [b] XX – PAO after lyophilization

2.2.6. Antioxidant assay of PAO and PAO-ME

A BioTek® PowerWave HT Microplate Spectrophotometer was used to analyze the antioxidant activity of the PAO and PAO-ME samples. The antioxidant assay was performed using an antioxidant assay kit (Item No. 709001) from Cayman Chemical. The kit contained the following reagents: assay buffer, metmyoglobin, chromogen, trolox, and hydrogen peroxide. 3mL of the provided assay buffer was diluted with 27mL of HPLC-grade water. The chromogen was prepared by adding 6mL of HPLC-grade water to 2,2'-azino-di-[3-thylbenzthiazoline sulfonate] (ABTS). Metmyoglobin was reconstituted via addition of 600µL of prepared assay buffer. Trolox solution was prepared by addition of 1mL HPLC-grade water to the given vial. 10µL hydrogen peroxide was diluted with 990µL HPLC-grade water to give a working solution of 441µM hydrogen peroxide. The assay required trolox standard solutions (7) made by serial

dilution and used to generate a trolox standard curve. The antioxidant activity of the PAO samples was measured and reported as comparable to a trolox solution with similar activity. Absorbances were read at 405 nm and 25^{o}C.

2.2.7. PAO-ME tested in "wired"-enzyme electrodes

BASi Bioanalytical Systems, Inc. glassy carbon electrodes (GCE, 3-mm diameter) were used to build the PAO-ME electrodes. The biosensors were used as working electrodes (WE) in three-electrode cells. The WE was coated with the most active PAO-ME obtained from each "wiring" procedure. Volumes of 100 □L of 10 mg of ME (IL-FAD/CU and HP30-CU, respectively, in phosphate buffer 67 mM pH 7.2, were placed on GCE and air-dried. Subsequently 50 □L 0.75% glutaraldehyde (used as a cross-linker) were placed on top and air-dried. The reference electrode was a standard calomel electrode (SCE) and the counter electrode was a platinum (Pt) wire. The electrochemical cell contained 75mL 67mM phosphate buffer pH 7.2 and it was used in cyclic voltammetry experiments run by an Epsilon Electrochemical Station from BASi. To assess the catalytic effect of PAO-ME, cyclic voltammograms were generated in the following conditions: the cell containing only the phosphate buffer determined the background current followed by the measurement of the current measured after benzylamine was added to the cell. Cyclic voltammetry was run between -0.5V and +0.8V for 10 cycles at a scan rate of 100mV/s. The 10th cycle was recorded and i_{max} values were extracted from the voltammograms. The linearity of the biosensor in response to amine concentration was determined by taking cyclic voltammograms at different concentrations of BA.

2.2.8. Fourier transform infrared (FTIR) spectra

FTIR spectra were recorded on a Bruker Tensor 27 instrument equipped with a Digi TectDLaTGS detector. Solid samples of lyophilized enzyme were placed on the ATR crystal and 32 co-added scans at 4 cm^{-1} resolution were taken on each sample.

2.2.9. Zeta potential and size measurements

The zeta potentials and the sizes of the ME samples were measured on a Zetasizer Nano-ZS (Malvern Instrument) equipped with a He–Ne laser (λ = 633 nm; scattering angle, 173°), using 1 g.L^{-1} phosphate buffer solutions of the samples.

3. Results and Discussion

PAO was "wired" through two different partial denaturation/renaturation procedures. In one emimBF_4 was used as denaturant, in the other procedure high hydraulic pressure was used as

denaturant.

Several parallel samples of PAO were denatured/renatured either without or in the presence of redox species relevant for PAO activity. Table 5 displays the modifier uptakes in the "wiring" procedures.

Table 5. Modifier uptake in the "wiring" procedures

ME Sample	FAD (%)	PLP (%)	LA (%)	$CuSO_4 \cdot 5H_2O$ (%)
IL-FAD	44.0	-	-	-
IL-PLP	-	10.0	-	-
IL-CU	-	-	-	90.4
IL-LA	-	-	~0	-
IL-FAD/CU	41.5	-	-	63.2
IL-FAD/LA	24.5	-	~0	-
IL-PLP/CU	-	16.4	-	53.6
IL-PLP/LA	-	7.1	87.2	-
HP60-FAD	45.6	-	-	-
HP60-PLP	-	18.9	-	-
HP60-CU	-	-	-	92.0
HP60-LA	-		12.0	-
HP30-FAD	43.2		-	-
HP30-PLP	-	18.2	-	-
HP30-CU	-	-	-	87.4
HP30-LA	-	-	3.7	-

The highest uptake was observed, in both procedures, for copper sulfate (90.4% for the IL modification, 92.0% and 87.4% for HHP modification at 60 min and 30 min, respectively). This makes sense if it is correlated to the fact that Cu^{2+} ions are cofactors for PAO and that TPQ formation (self-processing) requires copper and oxygen [22]. The active site of the enzyme is located near the center of each homodimer and is connected to the outside by an extensively hydrated channel. The consequence is that TPQ (prosthetic group of PAO) located at the bottom of a funnel-shaped cavity is widely exposed to the solvent and to copper II ions hydrated by the water present during exposure to IL. Several charged residues are distributed on the surface of this cavity among which D445, D385 (catalytic), L172, L468 [2]. One of the negatively charged residues (D445) is located very close to TPQ (less than 15 Å) making this cavity one of the most negative regions on PAO with the capacity to attract positively charged units (hence Cu^{+}).

Less anticipated was the poor uptake of PLP. It has been used as "wire" due to its

relatively close relationship to TPQ while being less expensive than the latter, and being the active form of vitamin B6 (toward potential synergistic effects in an enhanced PAO). In this respect, PLP enhanced notably the LA uptake and made assays of antioxidant activity of PAO-ME look as worth investigating since LA is known to act as a free radical scavenger and assist in repairing oxidative damage and regenerating endogenous antioxidant species. It was reported as a metal chelator and a chemopreventive agent as well [23]. We correlate the increase in LA uptake in the presence of PLP with the formation of an adduct between PLP and the reduced form of LA [24]. FAD was used in the preliminary study, to test the concept of "wiring" PAO through different routes since we used it successfully to modify two other redox enzymes (both flavoproteins). In both cases FAD provided enhancement and stabilization [15, 25]. Another reason for this choice was the fact that it presents a more enzyme/environment friendly alternative to the redox species typically used in enzyme wiring [9]. Beside the influence of PLP on LA uptake, the other binary combinations of modifiers show small decrease in each modifier's uptake compared to the modifications using single modifying species. This may be related to the fact that ME, renatured and likely with a slightly modified structure compared to the native PAO, is close to the latter in 3-D structure. The "wire" content in the modified PAO is comparable in the IL and HHP procedures with slightly higher values for HHP "wiring". No significant difference in modifier uptake was noticed between the HHP modification conducted for 60 min compared to the one conducted for 30 min. The same situation was reported in [26] for the case of glucose oxidase modified with ferrocene derivatives.

FTIR, DLS and zeta measurements of both PAO and PAO-ME were performed to study the impact of these procedures (IL and HHP, respectively) on PAO and to highlight possible interactions with the "wires".

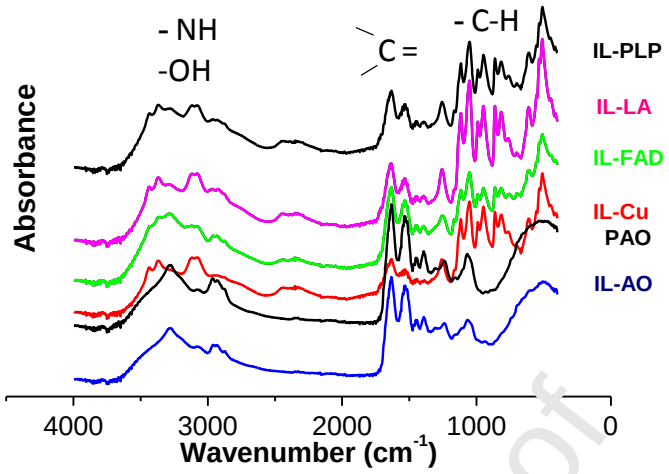

Figure 3. FTIR spectra of PAO, IL-AO*, IL-CU, IL-FAD, IL-LA and IL-PLP. IL-AO* - PAO exposed to IL without modifier present

For PAO and IL-PAO, two peaks found at 3360 and 3300 cm^{-1} can be respectively assigned to the stretching vibrations of –OH and $–NH_2$ groups. When "wires" are present, they shift to higher values. This may be linked to the interaction of the redox molecules and the PAO. Following IL modification (Figure 3), two intense peaks at 1637 and 1591 cm^{-1} are observed which correspond to ν (C=) vibrations of the TPQ ring [27]. Interestingly the intensity of these peaks decreased slightly for IL-PLP and IL-FAD and significantly for IL-CU. Compared to PAO, in PAO-ME new peaks appear 880 cm^{-1} and could be assigned to –C-H and be attributed to the addition of the modifier.

The zeta potential value of a solution of native PAO is above -12 mV. The zeta potential values for the IL-PAO and IL-PAO-ME solutions are -12 mV and - 9 mV, respectively. This change in zeta value following the addition of the modifiers shows that all modifiers interact with PAO. Furthermore, the size value for the native PAO was 10.9 nm (PDI ~ 0.4). "Wiring" of PAO led to a slight change in enzyme size. For the IL-CU, IL-FAD, IL-LA and IL-PLP, the measured sizes are 14.3 nm (PDI ~ 0.5), 10.7 nm (PDI ~ 0.5), 10.5 nm (PDI ~ 0.6) and 8.1 nm (PDI ~ 0.5), respectively. The highest increase for PAO-CU can be correlated with the highest uptake for this "wire" justified by PAO affinity for its cofactor. Employing FAD and LA did not result in a significant change in size. These modifiers may interact mainly via hydrogen bonds with PAO. When PLP, is used as "wire", the size is slightly decreased. The negatively charged

phosphate group of the PLP may interact with the copper cofactor of PAO via electrostatic interaction and induce compaction phenomena. Interestingly, in DLS data, there is only one population, which is present in the PAO-ME size results. All these results show clear interactions between PAO and all "wires", presumably of different types, according to structure and entrapped amount of each modifier.

Following HHP modification (30/60 min), the presence of CU, PLP, FAD or LA seems to have little impact on the enzyme structure since no alterations are observed in the FTIR spectra (Figure 4 for HHP-30 min and figure S1 for HHP-60 min). The zeta potential values for the PAO solutions are -9 mV (HP30), - 12 mV (HP60), and for PAO-ME they are - 8 mV (HP30-ME) and -11 mV (HP60-ME), respectively. The size value of the PAO submitted to high pressure is 14.7 nm (PDI ~ 0.4) and 22.5 nm (PDI ~ 0.3) for HP60 and HP30 respectively. For the HP30-CU, HP30-FAD, HP30-LA, HP30-PLP, the measured sizes are 14.3 nm (PDI ~ 0.5), 10.7 nm (PDI ~ 0.5), 10.5 nm (PDI ~ 0.6) and 8.1 nm (PDI ~ 0.5), respectively. For the HP60-CU, HP60-FAD, HP60-LA and HP60-PLP, the measured sizes are 19.4 nm (PDI ~ 0.3), 15.8 nm (PDI ~ 0.3), 24.4 nm (PDI ~ 0.3) and 10.2 nm (PDI ~ 0.4), respectively. For the HP30-CU, HP30-FAD, HP30-LA and HP30-PLP, the measured sizes are 18.3 nm (PDI ~ 0.4), 27.4 nm (PDI ~ 0.4), 25.8 nm (PDI ~ 0.4) and 10.2 nm (PDI ~ 0.4), respectively. The results for ME afforded by the HHP procedures showed like those for the ME from the IL procedure, that the modifiers became part of the 3-D structure of the renatured PAO.

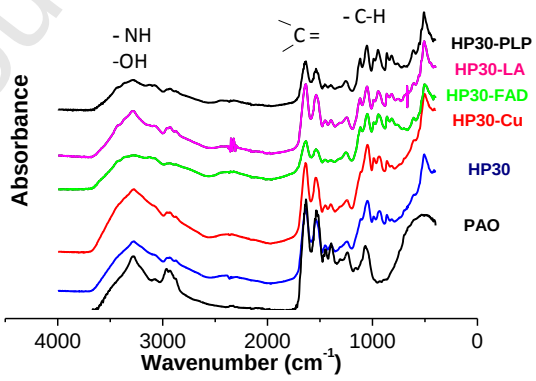

Figure 4. FTIR spectra of PAO, HP30, HP30-CU, HP30-FAD, HP30-LA, and HP30-PLP.

In the procedures used by us, PAO was first transiently exposed to either IL or HHP in the presence of redox active species. Upon removing the denaturant (IL/HHP) by dialysis of the mixtures used in the modifications, PAO refolds entrapping part of the modifier(s) within its

"wired" 3-D structure. The molecular "wiring" afforded thereby is expected to help efficient electron transfer in the resulting PAO-ME. PAO and PAO-ME were assayed and the results are presented in Tables 6 and 7.

Table 6. Activity of PAO-ME modified using emimBF_4

PAO-ME Sample	Activity[a] (IU)	Residual activity (%) (native)[b]	Residual activity (%) (modifier effect)[c]	~~Residual activity of PAO/PAO-ME after one year (%)~~
PAO native	$1.03*10^{-4}$	-	-	~~42.4~~
PAO-IL*	$9.04*10^{-5}$	87.8	-	~~8.2~~
IL-FAD	$5.68*10^{-5}$	55.0	62.9	~~68.7~~
IL-PLP	$1.78*10^{-4}$	171.0	189.4	~~105.0~~
IL-CU	$2.08*10^{-4}$	181.0	230.1	~~20.05~~
IL-LA	$4.44*10^{-5}$	42.9	49.1	~~170.0~~
IL-FAD/CU	$2.13*10^{-4}$	206.0	235.7	~~153.0~~
IL-FAD/LA	$8.88*10^{-5}$	85.9	98.2	~~134.0~~
IL-PLP/CU	$1.47*10^{-4}$	142.0	162.7	~~12.1~~
IL-PLP/LA	$1.51*10^{-4}$	146.0	167.1	~~1.5~~

PAO-IL* - PAO exposed to IL without any modifier present

[a] – PAO activity adjusted to reflect the loss due to lyophilization (70%)

[b] – Residual activity (native) $= \frac{\text{PAO–ME } activity}{\text{PAO native activity}} \times 100$ (%)

[c] Residual activity (modifier effect)) $= \frac{\text{PAO–ME } activity}{\text{PAO–IL activity}} \times 100$ (%)

Table 7. Activity of PAO-ME modified using high hydraulic pressure

PAO-ME Sample	Activity[a] (IU)	Residual activity (%) (native)[b]	Residual activity (%) (modifier effect)[c]	~~Residual activity after one year (%)~~
PAO native	$1.078*10^{-4}$	-	-	~~40.9~~
HP60	$9.540*10^{-5}$	56.87	-	~~55.9~~
HP60-FAD	$8.220*10^{-5}$	48.99	86.16	~~~100.0~~
HP60-PLP	$7.817*10^{-4}$	465.9	819.4	~~70.7~~
HP60-CU	$6.300*10^{-4}$	375.6	660.5	~~82.9~~
HP60-LA	$4.307*10^{-4}$	256.8	451.6	~~45.0~~
HP30	$1.801*10^{-4}$	107.4	-	~~186.0~~
HP30-FAD	$3.623*10^{-4}$	216.0	201.2	~~185.0~~
HP30-PLP	$1.315*10^{-3}$	783.6	729.8	~~7.9~~
HP30-CU	$2.651*10^{-3}$	1580.0	1471.4	~~10.4~~
HP30-LA	$8.457*10^{-4}$	504.1	469.4	~~62.6~~

[a] – PAO activity adjusted to reflect the loss due to lyophilization (70%)

[b] – Residual activity (native) $= \frac{\text{PAO–ME } activity}{\text{PAO native activity}} \times 100$ (%)

[c] Residual activity (modifier effect) = Residual activity (modifier effect) $= \frac{\text{PAO–ME activity}}{\text{HP 60/HP30 } activity}$ x 100 (%)

Tables 6 and 7 present the results of activity assays of PAO. The values have been adjusted to

reflect the loss in activity due to lyophilization. While the Worthington manual states 15-20% decrease in PAO activity following lyophilization [20], our tests done on the PAO used in the experiments resulted in a higher loss (70%). Transient exposure to IL without any modifier present and refolding resulted in a modest loss in activity (~13%) while the effect of HHP, without modifier present was different, depending on the compression time. While 30 min did not affect the activity, 60 min exposure to high pressure resulted in a higher decrease in activity (~43%) compared to the IL procedure.

The presence of a "wire" in the IL-ME affected activity differently. The presence of copper ions (intrinsic to PAO) and that of PLP (closely related to TPQ, prosthetic group of PAO) resulted in modified PAO with enhanced activity (171% and 181%, respectively) while the presence of FAD/LA resulted in re-natured PAO with lower activity (55% and 43%, respectively). We attribute this effect to the sterical hindrance in the renatured PAO-ME due to the relatively large amount and molecular size of FAD. When LA is present, the chelating effect of its carboxyl group (on the copper ions, cofactor) lead in our study to a decrease in PAO activity while Lunelli *et al.* [2] report PAO inhibition in LA presence. Additional Cu^{2+} in PAO-ME was beneficial to PAO activity when used in binary combinations as well while PLP did not result in such high enhancement when used together with another modifier present. When the effect of the "wire" was assessed compared to PAO –IL, the enhancement afforded by PLP and copper ions, respectively, were even higher (189% and 230%) while FAD and LA did not seem to make any difference when used in binary mixtures. ~~The denaturation in the presence of IL followed by renaturation affected the stability of PAO-IL compared to the native PAO. The stability of the latter was 3 times higher after 1 year in storage (at -20 ^{0}C) compared to PAO-IL. While the presence of a "wire" was beneficial to the stability of ME, surprisingly, copper ions (which provided the highest enhancement following modification) resulted in the highest decrease in activity after one year. We correlate this observation with several reported facts. Over time, the high number of copper ions could end up chelated by side-chains of residues present at the surface of the cavity and thereby disrupting the conformation at the active site and the access to TPQ.~~ ~~From Table 6 it appears that the lowest stability was observed when LA was present as "wire" which can be related to the chelating effect of carboxyl group (on the copper ions, cofactor) leading to PAO inhibition as reported by Lunelli *et al.* [2].~~

The HHP modification affected differently the PAO activity, depending on the

compression time. Shorter compression time (30 min) resulted in ME with higher activities, 100% compared to 57% for 60 min. The differences in residual activity afforded by exposure to HHP for 60 vs 30 min are consistent with results reported in the literature. Compression times of 2-60 min are reported, after which proteins degrade. [28, 29].

The presence of a "wire" was beneficial for PAO activity in all cases with the exception of FAD – 60 min. The same trend was observed if the effect of the "wire" was assessed separately. ~~A comparison of the stability of ME obtained by the HHP procedure using different compression times showed no advantage for the shorter compression time after a year in hstorage (at -20 ^{0}C). The presence of copper ions or PLP did not help stability over time. Can this be correlated with the high content of modifier (especially copper ions) which, in time, affects negatively the 3-D structure of the re-natured (modified) PAO?~~ In the case of copper ions as "wire", the discussion of the IL modification results applies herein as well. Transient exposure to IL seems to affect more the 3-D structure of PAO. We correlate these findings with the fact that while HHP (in the range used, 325 MPa) affects mainly hydrophobic bonds, to a much lesser extent ionic interactions, and even less hydrogen bonds [28], exposure to IL has a broader impact on non-covalent interactions and on solvation as well. Table 8 offers a comparison between the enhancement afforded to PAO by the two modification procedures. The HHP procedure results in a higher enhancement to PAO for all of the "wires" used, more so at shorter compression times.

~~The fact that IL-ME fares better in time (lower decrease in activity after a year) comparatively to the HHP modification may be attributed to a less extensive denaturation for the former since exposure to emimBF$_4$ affords less disruption in the structure of the native PAO comparatively to high pressure which may affect several types of secondary valence bonds in the folded enzyme.~~

Table 8. Comparison between the two modification procedures*

Modifier	Activity HP60/Activity IL (%)	Activity HP30/Activity IL (%)
-	105.5	199.2
FAD	144.7	637.9
PLP	438.8	741.6

$CuSO_4$	302.9	1274.0
LA	970.8	1909.9

- Activity of lyophilized, non-modified PAO was considered 100%

Since PAO is an antioxidant agent [30], the modified enzymes were tested for antioxidant activity as well. Table 9 summarizes the results presented as equivalent activities to standard trolox solutions [31]. In the IL modification the antioxidant activity of PAO did not seem to be affected by the modification or by the presence of any modifier in ME. PLP and LA imparted a very modest increase in antioxidant activity compared to the native and the non-"wired" ME. This observation was against our expectations since LA is a known strong antioxidant and PLP was reported as associated with increase in antioxidant activity of enzymes [32]. In the HHP procedure, the rearrangement after high pressure removal afforded a sizable increase in antioxidant activity. The nature of the "wire" does not seem to make a difference.

Table 9. Antioxidant activities of PAO and PAO-ME

Sample	Antioxidant activity (mM Trolox)	Sample	Antioxidant activity (mM Trolox)
PAO native	0.311	HP60	1.416
PAO-IL	0.310	HP60-FAD	1.935
IL-FAD	0.315	HP60-PLP	1.221
IL-PLP	0.323	HP60-CU	1.188
IL-CU	0.311	HP60-LA	0.117
IL-LA	0.311	HP30	2.455
IL-FAD-CU	0.310	HP30-FAD	1.513
IL-FAD-LA	0.345	HP30-PLP	1.675
IL-PLP-CU	0.327	HP30-CU	0.831
IL-PLP-LA	0.429	HP30-LA	1.123

The "wired" ME were tested in two amperometric biosensors using benzylamine (BA) as analyte. For the proof of concept, the most active modified enzyme obtained through each "wiring" procedure (IL-FAD/CU and HP30-CU) was used to prepare a biosensor, respectively. Both displayed catalytic effect for BA as shown in Table 10 (comparison between the second and first lines in the table). This is another proof that the molecular "wiring" was done

successfully through both procedures since none of the biosensors required a mediator. Thereby redox centers buried deep in the protein structure, hence not effective for e-transfer, became part of the e-transfer with the electrode. It also qualifies the molecularly "wired" PAO reported herein as providers of direct electron transfer (DET) between the enzyme and the electrode (traditional meaning of "wiring"). No quantitative relation can be established between the number of "wires" and the electron mediating activity since "wires" effective for mediating are only those accessible to both the prosthetic group of the enzyme and to the electrode. They are, most likely, those close to the surface of the enzyme (within electron tunneling feasibility), not those buried in layers of insulating protein. Due to this fact, a discrepancy between the activity of PAO-ME and the amperometric response could be correlated with a different impact of the action of the denaturant (IL/HHP) on the two steps involved in the catalytic reaction [15].

The linearity of the response seemed limited to 0-100 mM BA. More concentration intervals in the micromolar range need to be investigated for a definite answer concerning this characteristic of the response. This aligns with the objective of using such biosensors for medical applications or for food freshness. A biosensor for histamine reported in the literature displayed linear response on 10-200 □M while using mediation by a Os-bipyridinium modified polymer [33]. Yet another biosensor for fish meat freshness monitoring, using also a mediator, tested as sensing element amine oxidases from plant sources. It displayed poor operational stability and linearity limited to the nanomolar range [34].

Table 1. Amperometric response as a function of BA concentration

BA concentration (mM)	i_{max}-IL (□A)	i_{max} –HP (□A)
0	7.18	7.87
5	7.67	8.42
10	8.8	10.2
20	9.43	12
50	9.97	14.3
100	11.4	18.1
200	11.7	18.3

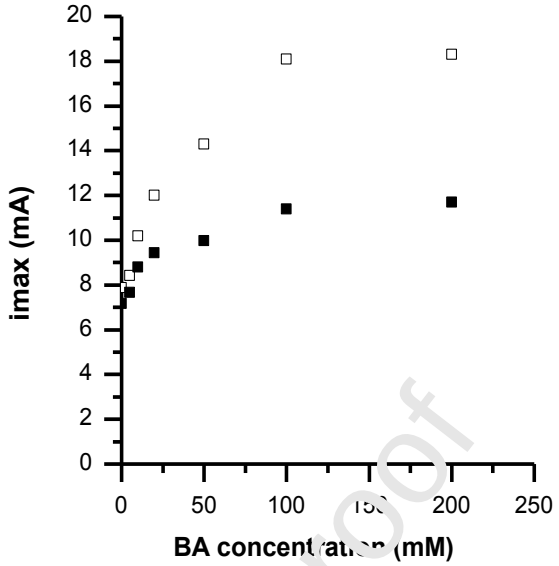

Figure 5. Amperometric current as a function of benzylamine concentration ■ biosensor with IL-CU and □ biosensor with HP30-CU

The response time for the two electrodes were 21s (IL-FAD/CU) and 18s (HP30-CU). The operational stability was characterized by less than 5% response loss for both electrodes after daily testing over 10 days, with storage in phosphate buffer 50 mM, pH 7.2, at 4 ^{0}C between tests. The "wiring" was done successfully through both procedures and none of the biosensors required a mediator.

4. Conclusions

Furthermore, it deserves noting that for amine oxidase to work in an amperometric biosensor, mediators are needed. [33,34], which are molecules which are toxic, hence their immobilization is necessary. This requires procedures which sometimes are labor intensive and/or may affect the activity of the enzyme (depending on choice of method). Therefore, the developed procedure in this work allows successfully overcoming this problem. Two different procedures are proposed for the molecular "wiring" of PAO. Both methods are environmentally friendly. One is using an IL, green reagent, to partially denature the enzyme, the other methods is totally reagentless, using HHP as partial denaturant, in the presence of modifying (redox) species. Upon removal of the denaturant, PAO folded entrapping modifier molecules in the 3-D structure of the protein and giving them the statute of molecular "wires". The choice of "wires"

is also an environmentally friendly one (differently from the Os and Ru complexes typically used in enzyme "wiring") and an enzyme friendly one, since the modifying species are related to PAO and/or nontoxic. The fact that IL is used not as medium in biocatalysis, as in most published reports, but as a means to modify an oxidoreductase is an approach first published by us in relation to two flavoenzymes [15, 25] and has never been tested or reported for a copper enzyme. The second procedure is a novel one, achieving "wiring" of a redox enzyme by using a purely mechanical, reagentless procedure.

Both methods afforded PAO with important residual activity. In this respect the presence of copper (cofactor) as a "wire" appeared to be the most beneficial to the ME. The refolded structures, although non-native, are stable ones. We correlate the stabilization and the enhancement in PAO activity with molecular mechanisms discussed in previous studies [14, 15, 25, 35-37].

The enzyme electrodes tested showed good promise, as proof of concept, for amperometric detection of amines without mediating species.

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Declaration of competing interests

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

x The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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