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ARTICLE

PEGylation as an efficient tool to enhance cytochrome c thermostability: a kinetic and thermodynamic study

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Cytochrome-c from equine heart was kinetically and thermodynamically investigated either in its native (Cyt-c) or PEGylated forms with different PEGylation degrees (Cyt-c-PEG-4 and Cyt-c-PEG-8). Maximum activities were observed at 80°C, and the irreversible deactivation was well described by first-order kinetics. The results of activity at different temperatures were used to estimate the activation energy of the catalysed Cyt-c reaction (E^* = 10.22 ± 0.40, 7.51 ± 0.06 and 8.87 ± 0.29 kJ.mol^{-1} for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8) and the standard enthalpy variation of enzyme unfolding (ΔH^o_U = 33.82 ± 4.92, 109.4 ± 13.1 and 58.43 ± 3.11 kJ.mol^{-1} for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8, respectively). The results of residual activity tests allowed estimating the activation energy (E^*_d = 50.51 ± 1.71, 72.63 ± 0.89 and 63.36 ± 1.66 kJ.mol^{-1} for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8), enthalpy ($\Delta H^‡$), entropy ($\Delta S^‡$) and Gibbs free energy ($\Delta G^‡$) of the enzyme irreversible denaturation. The higher enthalpic contributions of PEGylated conjugates and the increase in $\Delta G^‡$, compared to the native protein, indorsed the protective role of PEGylation. Negative values of $\Delta S^‡$ suggested the occurrence of an aggregation phenomenon by increasing the temperature, which was confirmed by circular dichroism. The estimated thermodynamic parameters suggest that PEGylated Cyt-c forms have enhanced thermostability, which would be of great significance for industrial biosensing applications.

Introduction

Horse heart cytochrome c (Cyt-c) is a heme protein involved in mitochondrial electron transfer with bio-electrochemical applications for hydrogen peroxide, polycyclic aromatic hydrocarbons (PAHs), and nitric oxide biosensing.[1] Cyt-c is recurrently applied in the catalysis of several chemical reactions including hydrogen peroxide reduction,[1] lipid peroxidation,[2] oxidation[3] and hydroxylation[4] of aromatic compounds. The peroxidase-like activity of Cyt-c has been demonstrated by the oxidation of various electron donors, among which are the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and PAHs.[5] This enzyme is considered an important redox biocatalyst since it is resistant to catalysis in organic solvents due to the covalent binding of the heme prosthetic group to the protein, it is stable in a wide range of pH (from 2 to 11) and cheap. However, there is a need to improve Cyt-c thermostability to increase its spectrum of applications as a biocatalyst and biosensor. One of the approaches followed to improve the thermostability of proteins is their chemical modification by bioconjugation.[6–8] This process is carried out by covalently binding a protective polymer to the biomolecule. The poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), poly(oligoethylene glycol methyl ether methacrylate) (POEGMA), poly(D,L-lactic-co-glycolic acid) (PLGA), poly(glutamic acid) (PGA), poly(N-isopropyl acrylamide) (PNIPAM), poly(N,N'-diethyl acrylamide) (PDEAM), polystyrene, and poly(ethylene glycol) (PEG), are some examples of polymers of biological and synthetic origin used for this purpose. PEG is a biocompatible polymer with low immunogenicity, antigenicity and toxicity, is soluble in water and other organic solvents and has high mobility in solution, which make it one of the polymers most used for bioconjugation.[9] In addition, among the few synthetic polymers, PEG is considered safe by the Food and Drug Administration (FDA) and European Medicine Agency (EMA).[10] The concept of protein PEGylation has been applied to several enzymes, leading to a new era of polymeric conjugate biocatalysts with improved properties. In the particular case of Cyt-c, PEGylation appears to be the first line option to enhance its thermostability.[11] Besides the improvement of thermostability, PEGylation improves several other properties, including its long-term stability, solubility in water, resistance to organic solvents and biocompatibility.[10,12] These conditions are

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very important taking into account that Cyt-c is used as a model biocatalyst in the biosensing field. The Cyt-c PEGylation was shown to kinetically stabilize the protein tertiary structure,[7] with the additional advantage of preserving the catalytic activity of enzymes at high temperatures.[8] Nevertheless, a thermostability study on the effect of PEGylation on this specific redox enzyme has never been reported.

The thermodynamic and kinetic studies can provide valuable information about the thermostability of enzymes at the operating temperature. Concerning the kinetic modelling of thermostability, a first-order deactivation reaction is usually adopted, describing its irreversible inactivation (denaturation), whose kinetics is usually expressed in terms of enzyme half-life ($t_{1/2}$).[13] On the other hand, the activation energy, Gibbs free energy, enthalpy and entropy of irreversible inactivation are important parameters widely reported in the literature to describe the thermodynamic behaviour of the enzyme denaturation phenomenon.[14]

In this work, the kinetic and thermodynamic parameters of the activity and thermostability of two PEGylated conjugates, Cyt-c-PEG-4 and Cyt-c-PEG-8, were investigated and compared with those of the native protein.

Materials and methods

Materials

Horse heart cytochrome c (Cyt-c, ≥ 95% purity), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS, ≥ 95% purity), hydrogen peroxide (99% purity), and hydroxylammonium chloride (99% purity) were obtained from Sigma-Aldrich/Merck (Darmstadt, Germany). High-purity methoxy poly(ethyleneglycol) N-hydroxysuccinimide ester (mPEG-NHS, 5 kDa) was acquired from Nanocs (New York, NY, USA). The aqueous medium used to perform the PEGylation reaction was composed of 100 mM potassium phosphate buffer. All other reagents were of analytical grade. All solutions were prepared using double distilled water, passed through a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus (Millipore/Merck, Darmstadt, Germany).

PEGylation reaction and purification of PEGylated products

The PEGylation reaction was carried out according to the procedures provided by the PEG derivative manufacturer.[15] Briefly, Cyt-c was dissolved in 0.1 M of sodium phosphate buffer and allowed to react with mPEG-NHS at room temperature (25±1°C) and constant magnetic stirring of 400 rpm. The experimental conditions of the reaction were pH 7.0, 1:25 protein:mPEG-NHS molar proportion and reaction time of 15 min. At the end, a 2.0 M hydroxylamine solution was added up to 1:10 (v/v) to stop the reaction as well as to avoid the formation of undesirable and unstable byproducts like conjugated esters.

After PEGylation and considering that the reaction is not complete, a complex media was obtained, composed of native Cyt-c, and two PEGylated forms, the Cyt-c-PEG-4 and Cyt-c-PEG-8. The PEGylated forms and unreacted Cyt-c were then separated by fast protein size exclusion liquid chromatography (SEC-FPLC), For that, a Superdex 200 Increase 10/300 GL column filled with crosslinked agarose-dextran resin (GE Healthcare Life Sciences, Chicago, IL, USA) was used in an AKTA™ purifier system (GE Healthcare). The column was equilibrated with 0.01 M potassium phosphate buffer containing 0.14 M NaCl, pH 7.4, and eluted with the same buffer at a flow rate of 0.75 mL.min^{-1}. Protein fractions with UV absorption at 280 nm, corresponding to the unreacted Cyt-c and PEGylated proteins, were stored at -20°C for further studies. Concentrations of Cyt-c and Cyt-c-PEG conjugates were determined using a calibration curve established in the SEC-FPLC under the conditions previously described. The PEGylation degree of Cyt-c conjugates was determined by SEC and eletrophoresis.[16] The molecular weights of the protein conjugates and, consequently, their degree of PEGylation were determined using a calibration curve with several proteins with known molecular weights.

Cyt-c activity assay

The enzymatic activity of Cyt-c was determined by the catalytic oxidation of 50 µM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in presence of 0.5 mM hydrogen peroxide.[5,6] The concentration of both non-PEGylated and PEGylated forms was 10 µM in 0.01 M potassium phosphate buffer (0.14 M of NaCl, pH 7.4). The reaction was started by the addition of hydrogen peroxide and followed by the absorbance increase at 418 nm.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of Cyt-c and PEGylated conjugates were acquired either in far-UV (190-260 nm) or near-UV (250-350 nm) range using a spectropolarimeter, model J-720 (Jasco, Tokyo, Japan). The final spectra were the average of 6 scans, followed by subtraction of the spectrum of 0.01 M potassium phosphate buffer (0.14 M NaCl, pH 7.4) obtained under the same conditions. Samples were placed in 1.00 mm optical length quartz cells with concentrations ranging from 13 to 15 µM. the spectra intensities (θ, mdeg) were converted to residual molar ellipticity ([θ], deg.cm^2.dmol^{-1}) by the following expression:

$$[\theta] = \frac{\theta}{10.C.l.n} \quad \textbf{(1)}$$

where C is the protein concentration expressed in M, l the optical length expressed in cm and n the number of amino acid residues of the protein (104 in this case).

The thermal stability of Cyt-c and its modified forms was also investigated by CD. Temperature was scanned from 30 to 90°C, at a rate of 1°C.min^{-1}, and back from 90 to 30°C to study the unfolding and refolding processes suffered by the enzyme, respectively. Samples with concentrations ranging from 2 to 4 µM were placed in 5.00 mm optical length quartz cells, and the intensities of ellipticity at 222 nm (θ_{222}, mdeg) were recorded throughout the experiment. Intensities of ellipticity were then converted to residual molar ellipticity ($[\theta]_{222}$, deg.cm^2.dmol^{-1}).

Determination of kinetic parameters

The Michaelis constant (K_m) and the maximum rate (v_{max}) of the enzyme-catalysed reaction were determined through double reciprocal (Lineweaver–Burk) plots of Cyt-c activity vs. ABTS concentration (0.025-0.300 mM). The turnover number (k_{cat}), defined as the maximum number of chemical conversions of substrate molecules *per* second that a single catalytic site executes at a given enzyme concentration, was calculated for Cyt-c and both PEGylated conjugates. The kinetic parameters were measured in triplicate and expressed as means ± standard deviations. All experimental data were analysed using the Prism 6.0 software (Graphpad Software, San Diego, CA, USA) for non-linear regression applied to Michaelis-Menten equation, and the determination coefficient (R^2) was used to compare the fitting performance. The Michaelis-Menten curves of Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8 are provided in **Fig. S1** at the Electronic Supporting Information (ESI).

Thermodynamic study

As described by Converti *et al.*,[17] the thermal inactivation of enzymes can be described by an enzyme unfolding equilibrium:

$E \leftrightarrows E_u$

where E and E_u are the folded and unfolded forms of the enzyme, respectively, with equilibrium constant K_U, coupled with a time-dependent irreversible denaturation, characterized by a first-order rate constant (k_d). At temperatures below the optimum temperature ($T < T_{opt}$), the equilibrium is shifted to the left side favouring the native, folded form of the enzyme, being the rate constant of the enzyme-catalysed reaction (k_0) described by the Arrhenius model. Conversely, when $T > T_{opt}$, the equilibrium is shifted to the right side favouring the unfolded form[18] and leading, after substitution of enzyme material balance in the K_U definition and Michaelis-Menten equation, to Eq. (2):

$$k_0 = \frac{A\exp(-E^*/RT)}{1 + B\exp(-\Delta H_U^o/RT)} \quad \textbf{(2)}$$

where E^* is the activation energy of Cyt-c-catalysed reaction, R the ideal gas constant (8.314 $J.K^{-1}.mol^{-1}$), A and B, the Arrhenius and an additional pre-exponential factors, and ΔH^o_U the standard enthalpy variation of the inactivation equilibrium previously described. If the unfolded form of the enzyme becomes predominant, what is observed while raising the temperature, Eq. (2) may be simplified to Eq. (3):

$$k_0 = \frac{A}{B}\exp\left(\frac{\Delta H_U^o - E^*}{RT}\right) \quad \textbf{(3)}$$

For the protein conjugates Cyt-c-PEG-4 and Cyt-c-PEG-8, the calculation of k_0 values is a challenge. Therefore, the proportionality existing between this parameter and the initial rate of product formation (v_0) was adopted. The E^* and ΔH^o_U were then estimated, either for native or PEGylated forms of Cyt-c, from the slopes of the straight lines of $\ln v_0$ vs. $1/T$, according to the Arrhenius equation and Eq. (3), respectively.

For the enzyme irreversible thermo-inactivation (denaturation), we used the transition state theory.[19] This process can be described by first-order reaction kinetics – Eq. (4):

$$v_d = k_d E \quad \textbf{(4)}$$

where v_d is the rate of enzyme denaturation, k_d the first order reaction rate constant, and E represents the concentration of enzyme active form. Introducing the activity coefficient (ψ), defined as the ratio between E and the enzyme concentration before exposition to different temperatures (E_0), k_d was estimated at each temperature from the slope of the straight line of $\ln\psi$ vs. time. The activation energy of irreversible enzyme denaturation (E^*_d) was then estimated from the slope of the straight line of $\ln k_d$ vs. $1/T$. The enthalpy of activation ($\Delta H^{\ddagger}$), Gibbs free energy of activation ($\Delta G^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$) of irreversible denaturation of either the native or PEGylated forms of Cyt-c were finally estimated, as described by Melikoglu *et al.*,[20] by Eqs. (5) to (7):

$$\Delta H^{\ddagger} = E_d^* - RT \quad \textbf{(5)}$$

$$\Delta G^{\ddagger} = -RT\ln\left[\frac{k_d\, h}{k_B\, T}\right] \quad \textbf{(6)}$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta G^{\ddagger}}{T} \quad \textbf{(7)}$$

where k_B and h are the Boltzmann and Planck constants, respectively.

The enzyme half-life ($t_{1/2}$) was defined as the time of exposure at a given temperature to reduce E to one half of E_0 and calculated by Eq. (8):[20]

$$t_{1/2} = \frac{\ln 2}{k_d} \quad \textbf{(8)}$$

Results and discussion

Structural effects of Cyt-c PEGylation

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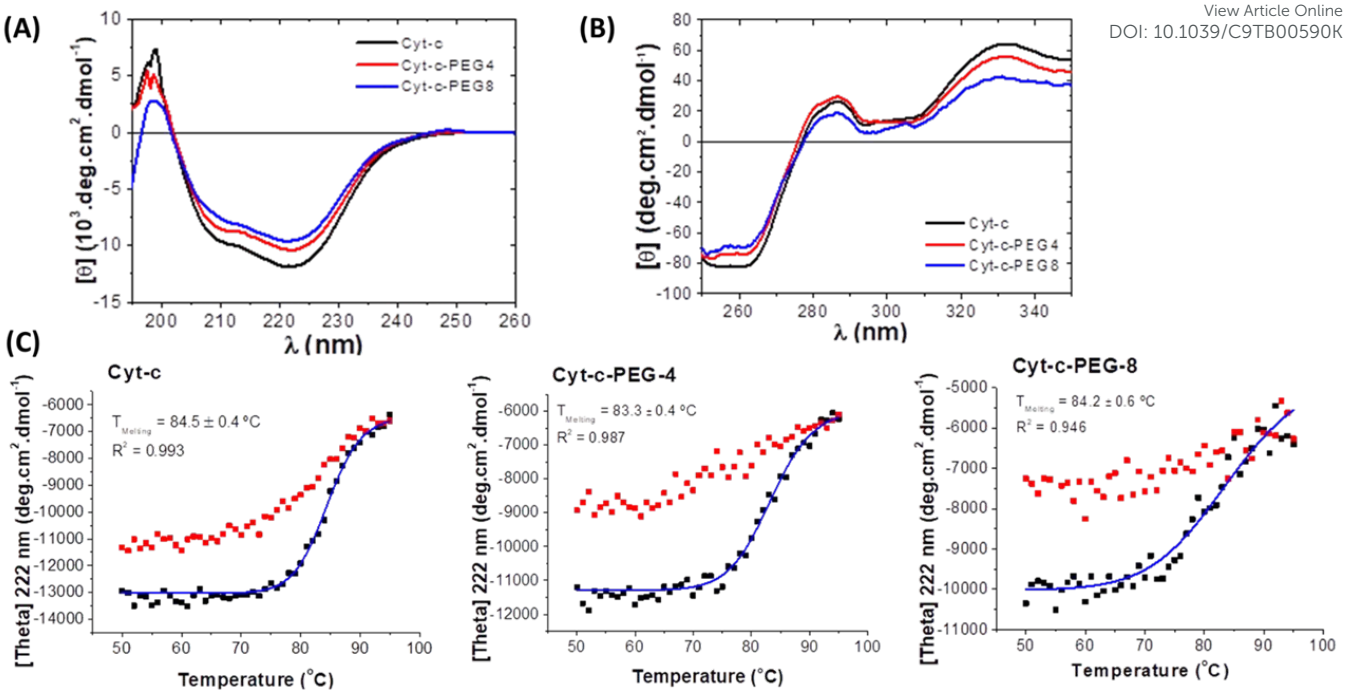

Fig. 1. (A) Far-UV and **(B)** Near-UV circular dichroism spectra of equine heart cytochrome c either in native form (Cyt-c, —) or PEGylated with 4 (Cyt-c-PEG-4, —) or 8 (Cyt-c-PEG-8, —) PEGs. Experiments were carried out with 13-15 µM of Cyt-c/Cyt-c-PEG-4/Cyt-c-PEG-8 in 0.01 M phosphate buffer (0.14 M NaCl, pH 7.4) at room temperature (25±1°C). **(C)** Thermal stability curves of unfolding (■) and refolding (■) processes of the same enzyme preparations. The stability curves were fitted by Hill equation (—).

Cyt-c was modified with mPEG-NHS (5 kDa) forming a heterogeneous mixture of unreacted protein, two PEGylated conjugates with different PEGylation degrees (number of grafted polymer chains) and PEGylation by-products (*e.g.* salts and PEG derivatives). Fast protein size exclusion liquid chromatography was applied to separate the different PEGylated forms and determine their PEGylation degree, *i.e.* the number of PEG chains attached to Cyt-c. The PEGylation reaction led to two purified fractions with four and eight mPEG chains *per* Cyt-c molecule (on average), similarly to other PEGylated conjugates reported in literature for this enzyme.[6,21]

To determine the PEGylation effect on Cyt-c secondary and tertiary structures, CD spectroscopy was employed.

The far-UV and near-UV circular dichroism spectra of unreacted Cyt-c and both PEGylated conjugates were evaluated being the main results depicted in Figs. 1A and 1B. In these figures, the characteristic secondary and tertiary structural fingerprints of Cyt-c are identified. The peak around 330 nm and the small peak at 300 nm are characteristic of tryptophan (Trp), those between 280 and 290 nm representative of tyrosine (Tyr) and the ones around 255–270 nm typical of phenylalanine (Phe). However, as previously observed by other authors,[7,21] the PEGylation reaction did not cause any significant spectral change either in the far (Fig. 1A) or the near (Fig. 1B) UV region, thus proving that the protein tertiary structure was maintained (Fig. 1B). Moreover, the different protein forms exhibited negative intensity bands centred at 208 and 222 nm and a positive peak in the 195-200 nm region of the spectrum, which are characteristic of a predominant α-helical secondary structure.

The Cyt-c thermal stability (folding/unfolding) was also investigated through circular dichroism (Fig. 1C). The unfolding curves showed similar behaviour for the three Cyt-c forms, and the proteins were found to be structurally stable, with melting temperature (*T*m) varying from 83.3 to 84.5°C. These similar values obtained reflect the reproducibility of the method for Tm determination. Above this temperature, all Cyt-c forms lost their tertiary structure. According to our results, PEGylation seemed not to increase the protein *T*m. Nevertheless, the results demonstrated that the unreacted Cyt-c has refolded more efficiently than Cyt-c-PEG-4, followed by Cyt-c-PEG-8, suggesting that the PEG chain interferes with the refolding process, probably by steric hindrance of the unfolded protein chain during refolding. We should keep in mind that, as explained in the Materials and Methods section, the investigation of thermal stability of Cyt-c and its PEGylated forms refers to a temperature scanning from 30 to 90°C, at a rate of 1°C.min^{-1}, and back from 90 to 30°C. Therefore, it cannot be assumed the stability at a fixed temperature will be the same for the two Cyt-c forms investigated. Differences were in fact observed between them in long-term residual activity tests performed in the temperature range 70–95°C (see later).

Kinetic parameters

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Table 1. Kinetic parameters of Cyt-c-catalysed reaction for the native cytochrome c (Cyt-c) or PEGylated conjugates, the Cyt-c-PEG-4 and Cyt-c-PEG-8. The catalytic oxidation of ABTS was performed in the presence of 0.5 mM of H_2O_2, at 25 ± 1°C and pH 7.4.

Protein	K_m (μM)[a]	v_{max} (nmol.min^{-1})[b]	k_{cat} (min^{-1})[c]	k_{cat}/K_m (min^{-1}.mM^{-1})	R^2
Cyt-c	36.00 ± 6.24	1.09 ± 0.05	0.91 ± 0.05	25.30	0.956
Cyt-c-PEG-4	73.24 ± 6.40	0.73 ± 0.04	0.61 ± 0.03	8.33	0.958
Cyt-c-PEG-8	85.21 ± 11.23	0.41 ± 0.02	0.34 ± 0.03	4.04	0.975

[a]Michaelis constant.; [b]Maximum reaction rate.; [c]Turnover number.

To demonstrate the influence of PEGylation on the kinetics of enzyme-catalysed reaction, the activity of unreacted Cyt-c and the PEGylated conjugates was evaluated in terms of their capacity to oxidize ABTS in presence of 0.25 mM hydrogen peroxide (Table 1). The main kinetic parameters of this reaction, namely the Michaelis constant (K_m), the turnover number (k_{cat}) and the maximum reaction rate (v_{max}), were calculated from the experimental results of activity collected varying the initial substrate concentration ($[S]_0$) from 0.025 to 0.300 mM. The nonlinear regression of the experimental data of reaction rate vs. the ABTS concentration revealed that all the three enzyme preparations followed, with good correlation (R^2 > 0.95), Michaelis-Menten kinetics. The kinetic parameters estimated for the three preparations varied in the ranges of 36.00 ± 6.24 μM < K_m < 85.21 ± 11.23 μM, 0.34 ± 0.03 min^{-1} < k_{cat} < 0.91 ± 0.05 min^{-1} and 0.41 ± 0.02 nmol.min^{-1} < v_{max} < 1.09 ± 0.05 μmol.min^{-1}. The slight decrease of k_{cat} and v_{max}, along with the increase in K_m observed for PEGylated conjugates in comparison with the native protein, indicates a negative effect of PEGylation on Cyt-c catalytic performance.

This effect was confirmed by the improvement of catalytic activity by decreasing the PEGylation degree, in that, Cyt-c-PEG-8 displayed lower k_{cat} but higher K_m than Cyt-c-PEG-4. This behaviour was already observed in previous works, where the decrease of the activity of several enzymes after PEGylation was reported.[22,23] Moreover, the PEGylation ability to stabilize the enzymes structure, even at high temperatures, compensates the decay of the kinetic parameters (*i.e.* k_{cat}/K_m ratio).[22,24,25] Laccase is an example where the PEGylation was shown to reduce the k_{cat}/K_m ratio for the ABTS oxidation.[24] Since in the present study the circular dichroism data excluded significant structural alterations of Cyt-c induced by PEGylation, these variations in the kinetic parameters may be attributed to (i) minor structural alterations in the heme environment[7] and/or (ii) to the loss of enzyme structural flexibility caused by the polymer binding.[26] In terms of oxidation of polyaromatic hydrocarbons (PAHs), the effect of Cyt-c PEGylation was different from that observed for other electron donors. For instance, in the specific case of tetrahydrofuran, the Cyt-c PEGylation was reported to induce a positive effect on the kinetics parameters, with an increase in the k_{cat}/K_m ratio from 4.1 min^{-1}.mM^{-1} to 132 min^{-1}.mM^{-1}.[8,27] Nevertheless, the oxidation of electron donors such as ABTS by Cyt-c-PEGylated proteins would still be advantageous taking into consideration the various beneficial effects of attaching PEG to the protein structure, for example its improved thermostability.

Thermodynamic study

Thermodynamic studies are important to understand the role of temperature on the enzyme activity. Here, the effect of the Cyt-c PEGylation degree on protein reversible unfolding and long-term thermostability was investigated using the same theoretical approaches recently applied to a large variety of enzymes with industrial potential, either in their free or immobilized forms.[28–31]

Thermal inactivation – Enzyme unfolding

The optimum temperature for Cyt-c activity was identified by performing activity tests in the temperature (T) range from 30 to 100°C. To minimize the influence of the irreversible denaturation on Cyt-c activity, only the initial values of enzyme activity (v_0) were taken into account. Cyt-c and Cyt-c-PEG conjugates proved to be active in the temperature range from 30 to 80°C, with an optimum temperature of 80°C, in accordance with literature.[1]

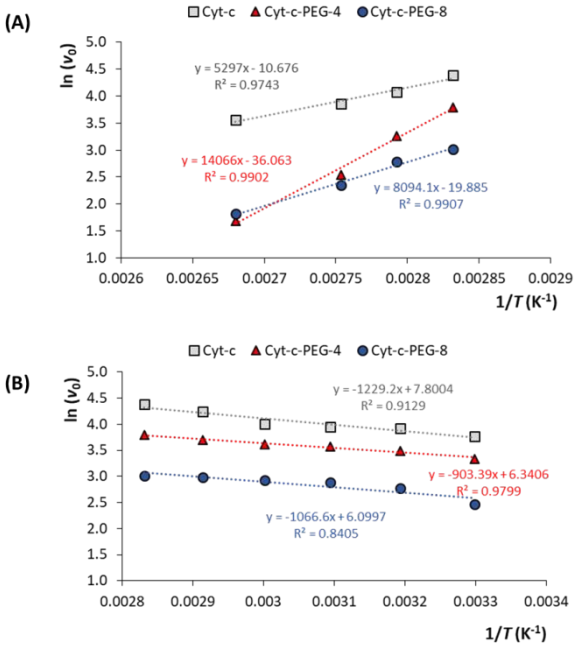

Fig. 2. Arrhenius-type plots of initial activity of Cyt-c and PEGylated conjugates with 4 (Cyt-c-PEG-4) or 8 (Cyt-c-PEG-8) PEGs attached, using 0.300 mM ABTS as a substrate. **(A)** temperature range 30-80◦C (T < Topt) and **(B)** temperature range 80-100◦C (T > Topt).

The semi-log plot of $\ln v_0$ vs. $1/T$ showed two tendencies. At temperatures below 80°C, a linear decrease was found (Fig. 2B), following the typical Arrhenius-type behaviour. Contrariwise, beyond 80°C, the opposite trend was observed (Fig. 2A), suggesting temperature-driven enzyme unfolding. The apparently irregular behaviour of Cyt-c-PEG-4 in Fig. 2A draws attention, in that, such a mild PEGylation seemed to promote protein unfolding, whereas the more marked one (Cyt-c-PEG-8) almost restored the folding status of native protein. This situation suggests that partial PEGylation might have occurred exclusively or predominantly on the same portion of protein molecule, thus deforming it. On the other hand, the more pronounced PEGylation may have evenly distributed the PEG molecules over the entire protein surface, almost cancelling that effect. From the slopes of the straight lines in the temperature ranges 30-80°C ($T < T_{opt}$) and 80-100°C ($T > T_{opt}$), the activation energies of the enzyme-catalysed reaction (E^*) and the standard enthalpy changes of enzyme unfolding (ΔH^o_U) were estimated for the native and PEGylated forms with satisfactory correlations ($R^2 > 0.84$ and > 0.97, respectively) and acceptably low standard deviations from the mean values of duplicate experiments (Table S1 at ESI). E^* was 15-36% higher for the native protein (10.22 ± 0.40 kJ.mol^{-1}) than for the PEGylated conjugates, suggesting a reduction of the energy of the transition state promoted by PEGylation. The very low E^* values estimated for all preparations indicate that little energy was required to form the transition state of ABTS oxidation, thus highlighting an effective oxidation capacity. Apparently, they seem to be inconsistent with the lower catalytic performance at mild temperatures ($T < T_{opt}$) of PEGylated conjugates, denoted by lower v_{max} and higher K_m values, compared to the native enzyme. This apparent inconsistency may be the result of (i) enzyme unfolding and/or irreversible denaturation interference (see the following section) on the starting activity, and/or ii) insufficient predominance of enzyme unfolding at $T > T_{opt}$, thereby making simplification of Eq. (2) to Eq. (3) not fully applicable.

PEGylation also increased the ΔH^o_U of thermal inactivation from 33.82 ± 4.92 kJ.mol^{-1} for the native protein to 109.4 ± 13.1 kJ.mol^{-1} for Cyt-c-PEG-4, which suggests an enzyme stabilization against unfolding at $T > T_{opt}$. Additionally, the higher ΔH^o_U value for Cyt-c-PEG-4 compared with Cyt-c-PEG-8 makes reasonable the hypothesis that excess PEGylation partially reduced this benefit. As suggested by Gaertner and Puigserver,[32] the entropic penalty related to the restricted motion of some amino acid groups on the protein surface due to the shell-like structure formed by coiled PEG chains may affect protein stability.

Thermostability – Irreversible enzyme denaturation

Long-term residual activity tests were carried out in the temperature range 70–95°C, whose results in terms of residual activity coefficient (ψ) are illustrated in the semi-log plots of Fig. S2 at ESI. The residual activities of Cyt-c and PEGylated conjugates followed the typical first-order decay due to denaturation already observed for other oxidoreductases.[33] For biosensors, the enzyme half-life ($t_{1/2}$) is a key parameter in terms of economic feasibility and resistance to thermal inactivation, which has become a required property for industrial applications of catalysts.[34,35]

The thermodynamic and kinetic parameters of the irreversible denaturation process are summarized in Table 2. As can be seen, $t_{1/2}$ progressively decreased, whereas the first order rate constant of Cyt-c thermo-inactivation (k_d) progressively increased with the increase in temperature, which means that its irreversible denaturation was more pronounced. Regarding the effect of PEGylation, the protective role of PEG conjugation is confirmed, since both Cyt-c-PEG-4 and Cyt-c-PEG-8 exhibited higher $t_{1/2}$ compared with the native protein. Additionally, the number of PEGs grafted to the protein greatly enhanced the enzyme long-term thermostability. $t_{1/2}$ for Cyt-c-PEG-8 and Cyt-c-PEG-4 were in fact about twice and 1.1-1.7-fold that of Cyt-c.

Semi-log plot of $\ln k_d$ vs. $1/T$ allowed estimating, with good correlation ($R^2 > 0.96$) and acceptably low standard deviations, the activation energies of denaturation (E^*_d) (Fig. 3), which were 50.51 ± 1.71 kJ.mol^{-1}, 72.63 ± 0.89 kJ.mol^{-1} and 63.36 ± 1.66 kJ.mol^{-1} for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8, respectively. The higher values of E^*_d obtained for PEGylated conjugates when compared to the native protein confirmed the thermostability promoted by PEGylation. Moreover, the higher E^*_d value for Cyt-c-PEG-4 compared to Cyt-c-PEG-8 indicated that excess PEGylation had a negative impact, not only on enzyme unfolding as previously discussed, but also on the conjugates thermostability. Comparison of these E^*_d values with those reported in the literature is challenging, mainly due to the large variability in the source and purity of the enzymes, as well as the substrates used in the thermostability assays.[34,36,37]

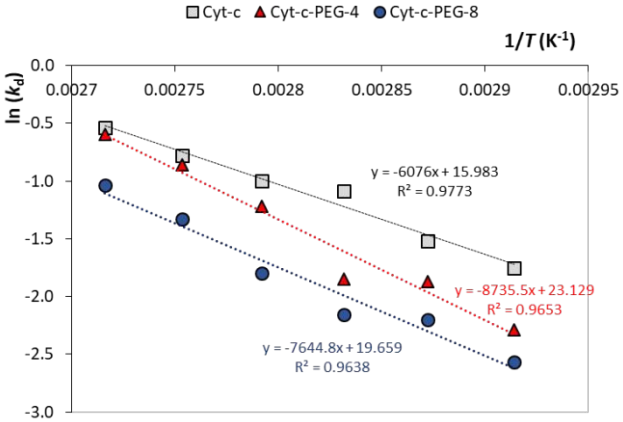

Fig. 3. Semi-log plots of the first-order denaturation constant (k_d) vs. the reciprocal temperature ($1/T$). The slopes of the resulting straight lines were used to estimate the activation energies (E^*_d) of irreversible inactivation (denaturation) of native cytochrome c (Cyt-c) and PEGylated conjugated with 4 (Cyt-c-PEG-4) or 8 (Cyt-c-PEG-8) PEGs attached.

Applying Eqs. (5–7), we calculated the activation enthalpy ($\Delta H^‡$), Gibbs free energy ($\Delta G^‡$) and entropy ($\Delta S^‡$) of Cyt-c and PEGylated conjugates denaturation (Table 2). These results indicate that the enzyme denaturation process is (i) exothermic ($\Delta H^‡ > 0$), (ii) not spontaneous ($\Delta G^‡ > 0$), and (iii) implies, at all

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Table 2. Thermodynamic and kinetic parameters of the irreversible thermal deactivation (denaturation) of native Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8.

	Thermodynamic and kinetic parameters					
	T (°C)	k_d (h^{-1})	$t_{1/2}$ (h)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta G^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol.K)
Cyt-c	70	0.173	4.00	47.65	101.13	-155.83
	75	0.219	3.17	47.61	101.97	-156.13
	80	0.339	2.05	47.57	102.19	-154.67
	85	0.369	1.88	47.53	103.43	-156.08
	90	0.459	1.51	47.49	104.25	-156.31
	95	0.587	1.18	47.45	104.98	-156.28
Cyt-c-PEG-4	70	0.101	6.84	69.78	102.66	-95.82
	75	0.154	4.49	69.74	102.98	-95.48
	80	0.157	4.41	69.69	104.45	-98.41
	85	0.295	2.35	69.65	104.09	-96.16
	90	0.422	1.64	69.61	104.50	-96.09
	95	0.551	1.26	69.57	105.17	-96.70
Cyt-c-PEG-8	70	0.077	9.05	60.50	103.46	-125.18
	75	0.111	6.26	60.46	103.94	-124.89
	80	0.116	5.97	60.42	105.33	-127.18
	85	0.165	4.19	60.38	105.82	-126.87
	90	0.264	2.63	60.34	105.92	-125.53
	95	0.352	1.97	60.30	106.55	-125.63

Note: $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$ values were calculated from E^*_d by Eqs. (5-7). E^*_d estimated from duplicate experiments was 50.51 ± 1.71 kJ mol^{-1}, 72.63 ± 0.89 kJ.mol^{-1} and 63.36 ± 1.66 kJ mol^{-1} for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8, respectively. Accordingly, all $\Delta H^{\ddagger}$and $\Delta S^{\ddagger}$ values for Cyt-c are affected by percentage errors with respect to the mean values listed in this table of ± 3.6% and ± 3.0-3.2%, respectively, those for Cyt-c-PEG-4 of ± 1.3% and ± 2.5-2.7%, respectively, and those for Cyt-c-PEG-8 of ± 2.8% and ± 3.6-3.9%. All k_d values estimated from duplicate experiments were subject to percentage errors in the range ± 0.4-5.1%, accordingly the $\Delta G^{\ddagger}$ values are affected by percentages errors in the range ± 1.3-1.6%.

tested temperatures (T = 70-95°C), a transition state with an unexpected more rigid structure than the reacting system ($\Delta S^{\ddagger}$ < 0), either for native or PEGylated forms. In particular, $\Delta H^{\ddagger}$ is an important thermodynamic parameter to consider, since it expresses the total amount of energy required to promote the enzyme denaturation,[13] which is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, hydrogen bonds, and van der Waals forces.[20]

Lower $\Delta H^{\ddagger}$ values at higher operating temperatures indicate that the enzyme thermo-inactivation was boosted. So, the higher enthalpic contributions of PEGylated conjugates compared to the native protein mean that higher energy is needed to denature the PEGylated conjugates, thereby endorsing the protective role of PEG coupling. The extent of the enzyme thermostability also depends on $\Delta S^{\ddagger}$, which expresses the amount of energy *per* temperature degree involved in the transition from a native to a denatured state.[13]

Negative values of $\Delta S^{\ddagger}$ like those estimated in this study suggest that denaturation of either native or PEGylated protein may have been contrasted by an aggregation process in which a few inter- and/intra-molecular bonds were formed.[38] However, whereas mild PEGylation (Cyt-c-PEG-4) seemed to have weakened such an aggregation, excess PEGylation (Cyt-c-PEG-8) seemed to restore it.

Thermal circular dichroism studies (Fig. 1C) revealed a change in protein conformation at temperatures around 75°C, suggesting that steric hindrance may have influenced the entropic variations. When an enzyme proceeds to the aggregated complex, the state of order of the system increases ($\Delta S^{\ddagger}$ < 0), reducing the consequent randomness degree of such a transition. Negative values of $\Delta S^{\ddagger}$ were also found for other redox enzymes[33], hydrolases[39] and proteases[40,41] at high temperatures. Finally, the $\Delta G^{\ddagger}$ combines both enthalpic and entropic contributions.[13] When $\Delta S^{\ddagger}$ < 0 and $\Delta H^{\ddagger}$ > 0, the denaturation process is not spontaneous ($\Delta G^{\ddagger}$ > 0), as it was found in this work. A negative value of $\Delta G^{\ddagger}$ is associated with a spontaneous process, meaning that the protein is thermosensitive. An increase in $\Delta G^{\ddagger}$, on the contrary, can be understood as an increase in the enzyme resistance to denaturation, *i.e.* increased thermostability. Even though the $\Delta G^{\ddagger}$ values listed in Table 2 are similar for the three enzyme preparations, the highest $\Delta G^{\ddagger}$ data were obtained for the PEGylated conjugates in the entire range of temperature investigated, and especially for Cyt-c-PEG-8, which is consistent with the thermoprotective role of PEG previously discussed. However, contrary to what was observed for reversible unfolding, no negative impact of excess PEGylation was detected for irreversible thermo-inactivation of the enzyme.

Conclusions

In this work, the kinetic and thermodynamic properties of PEGylated Cyt-c, a model redox protein, were determined. Maximum activity was observed at 80°C for Cyt-c and both PEGylated forms, Cyt-c-PEG-4 and Cyt-c-PEG-8. The half-life for PEGylated conjugates, as well as the thermodynamic parameters of the enzyme denaturation were estimated based on tests of the residual activity. The activation Gibbs free energy and enthalpy for PEGylated conjugates were higher than those for the native protein, consistently with the protective role of this polymer, which was shown to increase with temperature. All the uncertainties of the estimated thermodynamic quantities were very low, therefore the effects of PEGylation noticed in this study should be considered significant. Our results highlight the improved long-term thermostability of the PEGylated forms of Cyt-c that could be profitably exploited for future industrial applications, especially in the biosensing and pharmaceutical sectors.

Conflicts of interest

There are no conflicts to declare.

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PEGylation of Cytochrome-c preserves activity and increases thermal stability, favoring the protein application as a biosensor.

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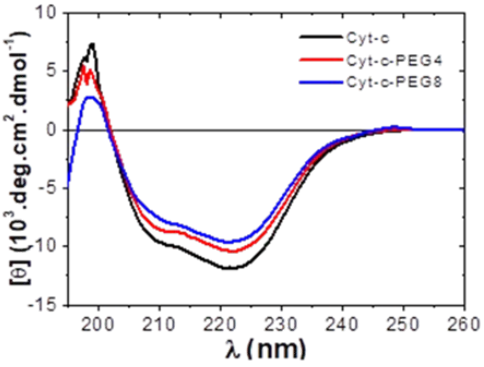
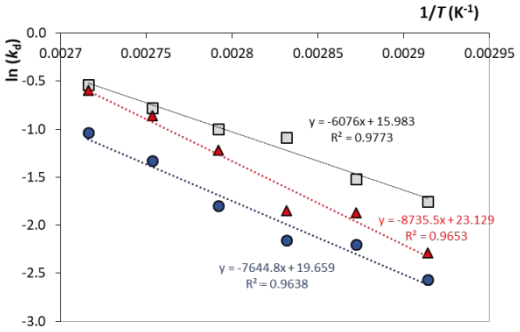


Far-UV circular dichroism spectrum shows.



Semi-log plots of the first-order denaturation constant (k_d) vs. the reciprocal temperature ($1/T$). The slopes of the resulting straight lines were used to estimate the activation energies (E^*_d) of irreversible inactivation (denaturation).