ISSN : 0974 - 7540 Volume 5 Issue 3

Research & Reviews On Electrochemistry

Full Paper

RREC, 5(3), 2014 [69-75]

A simple strategy for electrochemical study of proteins avoiding metallic groups

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ABSTRACT

A simple strategy for studying electrochemical properties of proteins or peptides using unmodified carbon paste electrodes was developed by cross-linking dopamine (DA) with BSA as a test protein. The BSA-5-Cys-DA adducts produced were evaluated by cyclic voltammetry, spectrofluorometry and first-derivative spectrophotometry in the visible range. An anodic peak was observed after protein isolation, not found with BSA samples only. This approach allow to overlap the sluggish kinetics of electron transfer exhibited by proteins with no metallic prosthetic groups near the electrode surface, providing thermodynamic and conformational studies by electrochemical techniques. A marked increased in fluorescence signal at 350 nm emission together with the appearance of an absorption signal at 370 nm have also arisen from the protein adducts. In this sense the covalent linkage between BSA with dopamine disclosed it as a dual candidate for both electrochemical and spectroscopic studies of proteins either in solution or immobilized on electrode surfaces.

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KEYWORDS

Protein tag;
Dopamine;
Cyclic voltammetry;
Electrochemical probe;
Spectrofluorimetry.

INTRODUCTION

Proteins are viewed as the ultimate target in many biological studies, as in protein structure, folding, unfolding and misfolding, protein dynamics, enzyme kinetics, and binding experiments[1], aiming applications ranging from chemistry and biochemistry, clinical diagnostics, pathology and immunology, to biotechnology and industrial targets. For this purpose a variety of techniques with different analytical methods from spectrophotometry[2], spectrofluorometry[3], light scattering[4], radioactive labelling[5], infrared reflection–adsorption spectroscopy[61, electron microscopy[7], ellipsometry[8], and cyclic voltammetry, among others, have been applied to identify proteins in solutions and to study their structure-function relationships. Among them, cyclic voltammetry is a powerful technique for inspection of electrode process, which reveals the faradaic as well as non-faradaic processes occurring within the interfacial region close to the electrode surface.

Nevertheless proteins often exhibit strong adsorption phenomenon on electrode surfaces, hindering elec-

tron-transfer rates at the electrodesolution interface[10]. The adsorption is affected by a number of factors, including temperature, pH, ionic strength, conformational state of proteins in solution and their bulk concentration. Nevertheless, some proteins as cytochromes[11], myoglobin and hemoglobin[12], superoxide dismutase and peroxidase[13], among other metalloproteins, have been largely studied as they exhibits prosthetic redox centers that make easier the electron transfer near the electrode surface. Proteins without metal redox centers in their structure, however, have been minor explored in protein electrochemistry[14], even though many efforts target to protein entrappment (nafion, hydrogels), membrane electrodes, redox mediators and electrode modifications have been reported.

The poor kinetic rate of electron tranfer viewed in proteins is due to burying of electroactive groups deeply within the overall structure, adsorption onto electrode surfaces of macromolecular species or denaturated forms of proteins themselves, and to unfavorable approaches to the electrode. Hence coupling the biological

component to a redox substance can cause an enhancement in the electrochemical current[15]. Furthermore, analytical performance of electrochemical biosensors has contributed to their versatility and usefulness making them prevail over other methods of signal transduction in biosensors[16]. Dopamine, 3,4-hydroxyphenyl ethylamine, DA, is an important neurotransmitter in biological systems and its deficiency is associated with Parkinson disease and schizophreniaS. Since DA can be easily oxidized[19], many electrochemical methods are commonly explored for its determination[20]. DA oxidation (Figure 1) has been previously demonstrated to modify proteins and kinetic enzyme activities by cross-linking of its reactive dopamine quinone with cysteine residues through a 5-S-Cys bound[21].

DA also exhibits both spectrophotometric and spectrofluorimetric properties with maxima absorption spectra at 298 nm[22] for oxidized form and fluorescence emission intensity at 315 nm[23]. In this sense dopamine can be viewed as an important biosensor as a dual candidate with displays both electrochemical and spectroscopic signals. In this work we have covalently attached dopamine to the test protein bovine serum albumin (BSA), as it has a single free thiol group at Cys-34 position[24], and carried out electrochemical and spectroscopic studies on the tagged protein. Then the goal of this work was to develop an alternative and simple technique for study electrochemical properties of proteins and peptides in general, specially those having no prosthetic metal groups.

EXPERIMENTAL

Reagents

N-ethylmaleimide (NEM), dopamine (DA), L-Cysteine (CySH), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, USA). All other chemicals were of the highest quality as possible as obtained, and all chemicals were used without further purification. Double distilled water was used for all preparations. Stock solutions of reagents were prepared by dissolving an accurate amount of each compound in water and then stored in the refrigerator in dark place. BSA were determined from its extinction coefficient at 279 nm (0.667 at 1%)[24]. NEM derivative of BSA was prepared by reacting NEM with BSA at a molar ratio of 5:1 in 0.1 M sodium phosphate buffer pH 7.45 for 3 days at 4^0 C upon continuous stirring, and the product isolated by dialysis and gel filtration in the same buffer.

Free thiol determination

Total thiol groups was determined using Ellman´s reagent[25]. After additon of 10-150 μL samples in the buffer, 200 μL of 10 mM 5,5´-Dithiobis(2-nitrobenzoic acid), DTNB, was added, and the color was allowed to develop for 20 min. The absorbance was spectrophotometrically measured at 412 nm. The values were calculated by comparison with CySH standard solu-

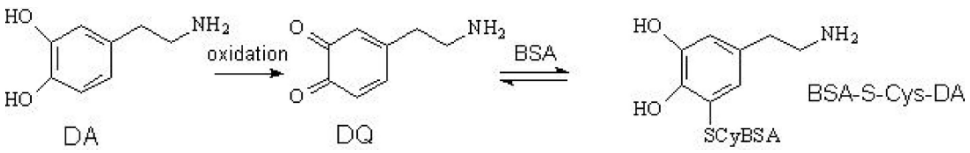

Figure 1 : The oxidation of DA to DA quinone and the resultant conjugation with thiol groups

tions (10-150 μM).

Cross-linkage of BSA with DA

The binding of dopamine with BSA was carried out mixing 0.16 mM of protein with 8 mM of dopamine in the buffer at 37 ºC for 2 h and in aerated buffer, to ensure the oxidation of dopamine to form dopamine quinone. The samples were dialysed with a cellulose membrane (Spectrapore, MWCO 6000-8000, Spectrum Laboratories, Inc, CA) during 24 h with a 2 h-period of buffer changes. Afterwards, the content of bags was gently withdrawn to avoid oxidation and protein denaturation, and the mixture was applied to a small Sephadex G-25 column (0.7 x 15 cm) equilibrated with phosphate buffer. The solutions eluted with the same buffer under the chromatographic peaks corresponding to major products were collected separately (1.5 mL fractions) and immediately kept at 5 ^{0}C before electrochemical and spectroscopy experiments.

Cyclic voltammetry

Electrochemical experiments were performed in a standard three-electrode cell using a PG39MCSV potentiostat:galvanostat for measurements (Omnimetra Instr. Científicos Ltda, RJ, Brazil), and 0.1 M sodium phosphate buffer pH 7.45 as electrolyte solution. An Ag/AgCl was used as reference electrode, and a Pt wire as auxiliary electrode. The work electrode was constructed by a 70:30 mixture of 20 μm graphite (99.9% purity, Fluka, St. Louis, USA) with Nujol (Mantecorp, RJ, Brazil). The potential was scanned from -300 to + 600mV employing 100 mVs^{-1} scan rates. Each single experiment was performed more than three times and at room temperature.

Spectroscopy studies

The change in the optical properties of the protein adducts have been studied using first derivative spectrophotometry and fluorescence spectroscopy. The absorption spectrum of the sample solutions contained DA or DA in the presence of BSA was measured against the reference buffer solution with a 2 nm slit width using 10 mm light-path cell. The spectral data were obtained with a S22 instrument (Biochrom Ltda, St. Albans, UK) and stored in a computer. The first derivative absorption spectra were obtained with a 11-point Savitzky-Golay derivative function (1 nm wavelength interval)

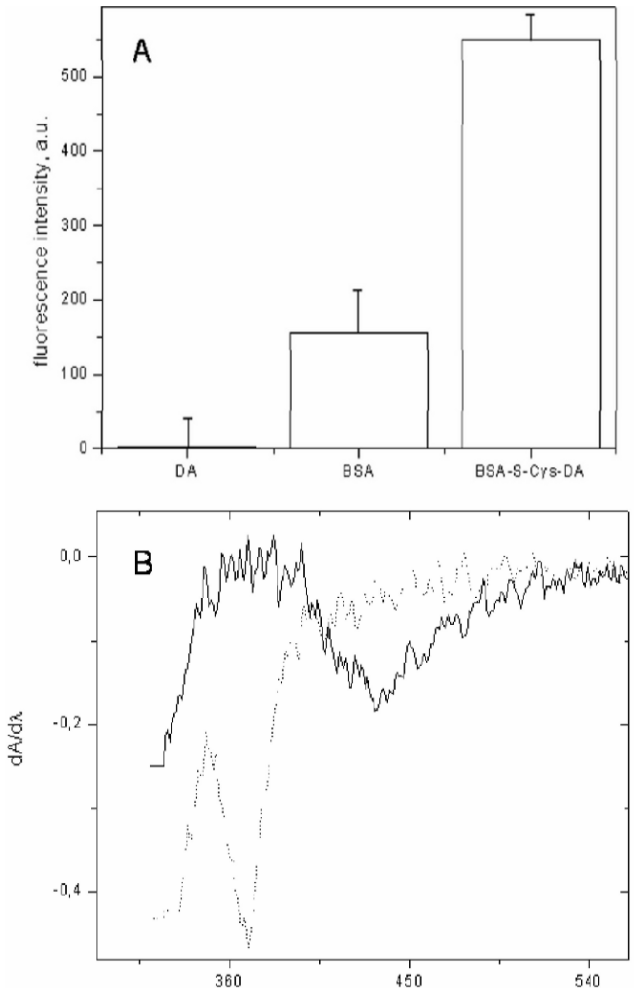

Figure 2 : A Titration of sulphydryl groups with DTNB in the presence of CySH (○), BSA (□), BSA complexed with DA (△), and BSA complexed with NEM (▽), in 0.1 M sodium phosphate buffer pH 7.45

with the statistical package Origin 8.0 (OriginLab Co, Northampton, MA, USA). All data were collected in triplicate and at ambient temperature. The fluorescence measurements were carried out in 0.1 M sodium phosphate buffer containing 5 μM DA, BSA or eluted adduct of DA with BSA (BSA-S-Cys-DA) samples, in 10 mm square quartz cells using a Varian Cary Eclipse (Varian Inc, Palo Alto, CA, USA). Emission spectra were scanned from 250 to 500 nm, and the peak intensity at 279 nm were determined. A blank measurement recorded prior to protein addition was substracted from the emission spectra of the mixtures.

RESULTS AND DISCUSSION

Figure 2 presents the resulted from DTNB titration

of thiol groups in cysteine (CySH), BSA, BSA complexed with N-ethylmaleimide (BSA-NEM) and BSA complexed with dopamine (BSA-S-Cys-DA). The total thiol groups found from these data were 1.2 ± 0.2 and 0.7 ± 0.2 for CySH and BSA, respectively, revealing the equivalency of the number of sulfhydryl groups for each molecule. The small free thiol content found for BSA would be due to its single buried Cys-34 residue in a shallow crevice in domain I, as compared to the CySH freely available in solution.

Both complexes with BSA did not present a measurable thiol content, suggesting a loss of binding when the sulfhydryl groups of CySH and BSA were blocked with DA and N-ethylmaleimide, respectively. The reaction between DA quinone, produced from auto-oxidation in the presence of oxygen, with CySH, resulted in the formation of 5-S-cysteinyl-DA (Figure 1)[26]. In this sense DA can oxidizes to form reactive quinones that bind to nucleophilic sulfhydryl groups on protein cysteinyl residues[27]. Figure 3 presents the voltammogram for DA (a) and the produced adduct of DA with Cys (DA-5-S-Cys) (b) obtained at the unmodified carbon paste electrode.

The anodic peak potential (Epa) for oxidation of the DA at the electrode surface was 159 mV, a value close to 173 mV reported by Shen and Dryhurst[27].

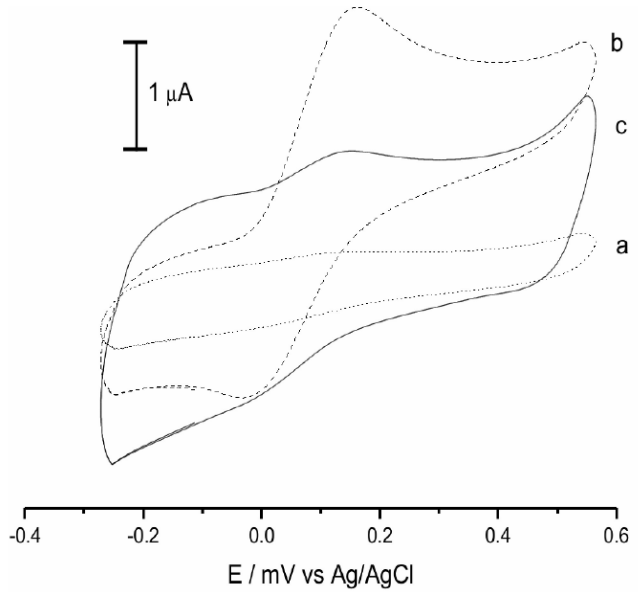

Figure 3 : Cyclic voltammogram of DA (a) DA with CySH in the presence of equimolar concentration of AA (b), and DA-5-S-Cys (c) in 0.1 M sodium phosphate buffer pH 7.45. Scan rate: 100 mV/s

The cathodic peak found for free DA (Figure 3, curve a) can not be depicted from DA interaction with CySH, suggesting the effectiveness of the oxidation reaction and cross-linking between CySH and DA.

We have used ascorbic acid (AA) at equimolar concentration with DA to test a plausible inhibition of BSA-S-Cys-DA oxidization reaction. AA is a strong reducing agent with ability to reduce quinone species and thus prevent nucleophilic reactions[28]. As can be shown in Figure 3 (curve b), the cathodic peak arisen for DA preincubated with equimolar amounts of AA almost overlapped the original DA profile (Figure 3, curve a), thus indicating the antioxidant ability of ascorbic acid in preventing the aminochrome formation from DA oxidation[29]. Furthermore, there was a shift of Epa values to higher positive potentials in the absence of AA. This change can be explained by specific interactions between the DA and CySH molecules, suggesting a decrease in the kinetic rate transfer together with a higher energy barrier for the electron transfer on the DA-5-S-Cys complex[30]. In short AA prevented the oxidative pathway of DA cross-linkage with the protein.

Purification procedure

After an extensive dialysis following gel filtration, the samples were eluted following cyclic voltammetry. Figure 4 shows the elution profile of BSA-S-Cys-DA and DA only based on optical density data. Fraction 3 showed an increase in the absorption at 279 nm (where BSA shows maximum absorption) indicating the elu-

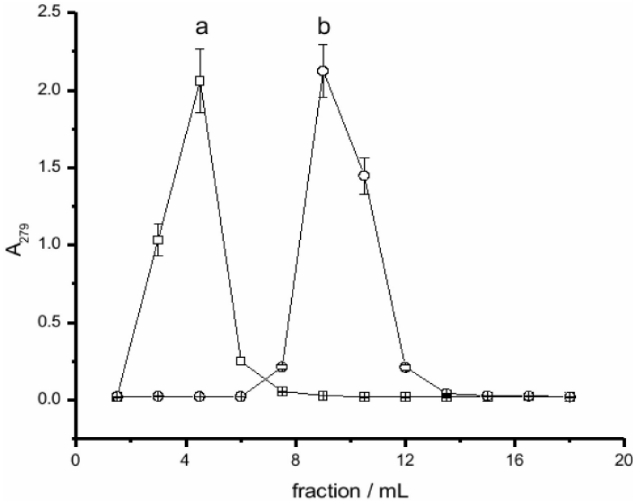

Figure 4 : Elution profile of BSA-S-Cys-DA (a) and DA (b) in Sephadex G-25 after dialyzing with 0.1 M sodium phostate buffer pH 7.45 during 24 h

tion of the labelled BSA (1 mL/fraction). As a comparison free DA was eluted after the sixth fraction collected (curve b, Figure 3). In this sense the freely available DA was effectively separated but the coupled protein and the uncoupled protein could not be distinguished by optical density.

The results obtained from electrochemical data of BSA-S-Cys-DA are shown in Figure 5. BSA does not show any significant peaks (Figure 5, curve a) but when tagged with DA it showed an intramolecular electron transfer taking place between BSA and DA (Figure 5, curve c) at an anodic peak potential near the Epa value found for free DA (Figure 5, curve b). Moreover the tagged protein presented higher current values as compared to untagged BSA, but smaller than those presented for free DA (Figure 5). This fact may be due to the steric hindrance for the large protein. [14] As obtained for DA-5-S-Cys (Figure 3, curve b), the oxidation process is not accompanied by a meaning reduction wave, which indicates that the oxidation reaction is totally irreversible, in opposition to the quasi-reversible DA oxidation shown in Figure 5 (a) and reported elsewhere[31]. In order to assess the influence of electron-transfer kinetics on the voltammetric behavior of the BSA-S-Cys-DA complex, we have determined the electrokinetic parameters from the relation of Laviron[32]. According to Laviron´s treatment for an irreversible electrode process, the standard heterogeneous rate constant for the electron transfer, k^o, and the charge transfer coefficient, α, can be obtained in a straightforward manner from the Eq. (1) below[32]:

$$E_p = E^o + (2.303\,RT/\alpha nF)\log(RT\,k^o/\alpha nF) + (2.303\,RT/\alpha nF)\log v \quad (1)$$

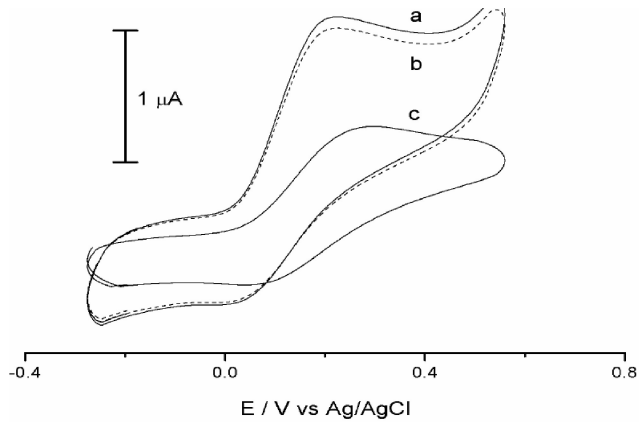

Figure 5 : Cyclic voltammogram of BSA (a), DA (b) and BSA-S-Cys-DA (c) in 0.1 M sodium phosphate buffer pH 7.45. Scan rate: 100 mV/s

where n is the number of electron transferred, v is the scan rate and E^o is the formal redox potential. Other symbols have their usual meanings. Thus the value of αn can be easily calculated from the slope of Ep versus $\log v$, and the value of k^o can be determined from the intercept if the value of E^o is known. The value of E^o can be obtained from the intercept of the Ep versus v curve by extrapolating to the vertical axis at v=0. With this treatment it was found k^o values of 1.5 ± 0.3 and 1.2 ± 0.5 s^{-1} for DA and BSA-S-Cys-DA, respectively, suggesting no meaning differences in the kinetic transfer rate between the free DA and the cross-linked protein with DA. Furthermore k^o values determined up to 12 days after produced BSA-S-Cys-DA did not showed any mean differences, suggesting a good stability of immobilized DA.

Spectrofluorimetry of BSA-S-Cys-*DA*

Dopamine is known to exhibit a fluorimetric spectra between 300-350 nm[23]. In this sense we carried out spectrofluorimetry runs aiming to study spectroscopy properties from the BSA-S-Cys-DA linkage. The data obtained from 250 to 500 nm emission of 5 μM samples are shown in Figure 6 (panel A).

DA shows maximum emission at 315 nm when excited at 279 nm[23], whereas BSA presented a λ_{max} at 342 (emission), mainly due to the band of tryptophan fluorescence. After covalently bound to the protein, the emission wavelengths shifted to the higher value of BSA but with an enhanced fluorescence intensity (Figure 6A). This change in the emission maximum can be explained

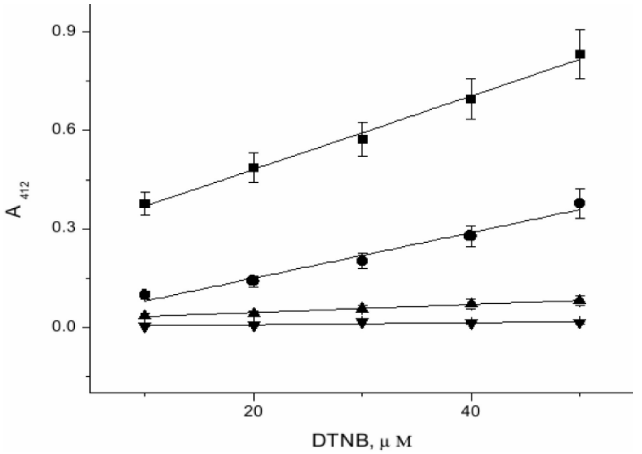

Figure 6 : A – Fluorescence intensity for DA (a) BSA (b), and BSA-S-Cys-DA (c) at 348 nm emission in 0.1 M sodium phosphate buffer pH 7.45. B – first-derivative spectrophotometry for DA (continuous line) and BSA-S-Cys-DA (dashed line)

by to the change in the environment of the oxidized quinone on binding to the protein when compared to free DA in solution. This suggests that the DA molecules have been effectively bound to the BSA molecules. Denaturation of BSA-S-Cys-DA induced with 6 M urea during 5 h also denoted a marked increase in the amount of fluorescence signal, higher than the value obtained for denaturated BSA only (data not shown). This result avoid the increase in fluorescence signal obtained as due to BSA denaturation induced by the cross-linking only.

First-derivative spectroscopy of BSA-S-Cys-DA

The absorption spectra of BSA is known to produce no changes in optical density in the visible range. The spectra of dopamine, however can present a slightly optical density due to oxidation reactions and intraciclyzation of the molecule[33]. In this sense first-derivative spectroscopy provides a means for presenting spectral data in a potentially more useful form than zero order, untreated data, disclosing minor details that are not presented in the primitive spectra[34]. Figure 6B presents the results from the spectrophotometric data for DA and BSA-S-Cys-DA. Mean changes in the first derivative spectrum for the complex are shown with a negative peak at 370 nm, as compared as the minor changes in DA sprectrum due to its oxidation (432 nm). This result corroborates with the electrochemical and fluorimetric data found for BSA-S-Cys-DA complex and reveals another spectroscopy property for the adduct.

From our results we conclude that DA mediated an intramolecular electron transfer in BSA and amplified the electrochemical signal for its detection. The significant changes in their redox as well as optical properties on binding to BSA can be used for biomolecular detection, structure-function relationship of proteins, and for biotechnology purposes involving both protein and peptide studies with different manners. As DA mediated the electron transfer on binding to the protein molecules it also can be used for bioelectrocatalysis. In this report, we have used BSA as a test protein but other proteins and enzymes with non-metallic redox centers but exhibiting free thiol groups (e.g., cathepsin B, protein dissulphide isomerase and α2-macroglobulin), as well as natural or synthetic peptides exhibiting free thiol groups, would be used in biosensor applications with greater efficiencies (signal), and using carbon paste electrodes without any modification. Moreover proteins without free thiol groups but under mild reducing conditions of their dissulphide bonds would be used in the same way. Since carbon paste electrodes are inexpensive, very facile to construct and may be adapted to almost any sensor device (large electrodes to capillar electrophoresis and electrochemical detection-HPLC), the protein-5-Cys-DA cross-linking would open up new possibilities for protein electrochemistry, together with its dual electrochemical and spectroscopical signals capability.

ACKNOWLEDGEMENTS

This work has been supported by the brazilian Research Foundation of the State of Minas Gerais (FAPEMIG), grant no. CBB- APQ-01920-11.

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