Talanta 147 (2016) 253–260

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fluorescence of the Flavin group in choline oxidase. Insights and analytical applications for the determination of choline and betaine aldehyde

E. Ortega [a], S. de Marcos [a], I. Sanz-Vicente [a], C. Ubide [b], M. Ostra [b], M. Vidal [b], J. Galbán [a,*]

[a] Analytical Biosensors Groups, Analytical Chemistry Department, Faculty of Sciences, Aragon Institute of Nanoscience, University of Zaragoza, 50009 Zaragoza, Spain

[b] Departamento de Química Aplicada, Facultad de Química, Universidad del País Vasco, Manuel de Lardizábal 3, 20018 San Sebastián, Spain

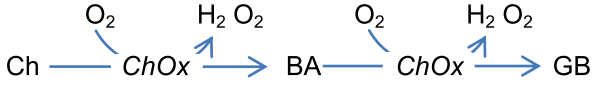
ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form
18 September 2015
Accepted 24 September 2015
Available online 30 September 2015

Keywords:
Choline oxidase
Intrinsic fluorescence
Flavin Adenine Dinucleotide
Choline determination
Betaine aldehyde

ABSTRACT

Choline oxidase (ChOx) is a flavoenzyme catalysing the oxidation of choline (Ch) to betaine aldehyde (BA) and glycine betaine (GB).



(1)

In this paper a fundamental study of the intrinsic fluorescence properties of ChOx due to Flavin Adenine Dinucleotide (FAD) is presented and some analytical applications are studied in detail. Firstly, an unusual alteration in the excitation spectra, in comparison with the absorption spectra, has been observed as a function of the pH. This is ascribed to a change of polarity in the excited state. Secondly, the evolution of the fluorescence spectra during the reaction seems to indicate that the reaction takes place in two consecutive, but partially overlapped, steps and each of them follows a different mechanism. Thirdly, the chemical system can be used to determine the Ch concentration in the range from 5×10^{-6} M to 5×10^{-5} M (univariate and multivariate calibration) in the presence of BA as interference, and the joint Ch+BA concentration in the range 5×10^{-6}–5×10^{-4} M (multivariate calibration) with mean errors under 10%; a semiquantitative determination of the BA concentration can be deduced by difference. Finally, Ch has been successfully determined in an infant milk sample.

1. Introduction

The intrinsic spectroscopic properties of enzymes are among the most important tools for investigating the kinetic mechanisms of reactions where they take part and for obtaining information about their structural/conformational alterations. Much of this information can be derived from the UV fluorescence spectra due to several aminoacids, such as tryptophan [1,2] and, more interestingly, some additional absorbing or fluorescent groups. In this context, flavoenzymes are particularly well positioned, especially those containing FAD [3].

Free FAD can be found in two main redox forms (oxidized and reduced) and both of them in different acid/base forms. When FAD takes part of the active center of an enzyme, its chemical properties can dramatically change [3–5]. For example the hemi-reduced oxidation state (semiquinone, FAD.H), scarcely found isolated in solutions, is easily stabilized in several flavoenzymes [6,7]; in addition, the acid/base dissociation constants can also change from one enzyme to another. From the spectroscopic point of view, the molecular absorption spectra of acid/base or redox forms of flavoenzymes barely change from those of FAD in solution. However, their fluorescent properties are highly affected by the specific environment surrounding a particular enzyme. This is due not only to the quenching produced by some molecular groups near to FAD, but also to structural reasons. It has been demonstrated that FAD in solution can exist in two different conformations [8,9]: open (fluorescent) and stacked (non-fluorescent). Depending on the environment of a particular enzyme, one of these conformations will predominate.

During enzymatic reactions, flavoenzymes move between oxidized and reduced (or semiquinonic) forms, so their spectroscopic

* Corresponding author.
E-mail address: jgalban@unizar.es (J. Galbán).

http://dx.doi.org/10.1016/j.talanta.2015.09.060
0039-9140/

properties change during the reaction. Such kind of changes can be analytically used, direct or indirectly [10,11], for determining the substrates involved in the corresponding reactions. However, starting from the first papers reported by Wolfbeis and Trettnak [12] this has mainly been limited to the determination of glucose using glucose oxidase [13–15]. There are several reasons for this: (1) as it has been indicated, the FAD fluorescence quantum yield cannot be predicted so it is necessary to carry out previous tests to evaluate this possibility for each particular enzyme; (2) because an excess of the substrate, compared to the enzyme, is generally used, the fluorescence intensity experiments some changes during the reaction and it is not easy to relate those changes in the experimental signal to the substrate concentration.

Choline oxidase (ChOx) is an enzyme that catalyzes the oxidation of choline (Ch) or betaine aldehyde (BA, also known as glycine betaine aldehyde) to glycine (GB, also known as glycine betaine) in bacteria. The mechanism has been studied by Gadda et al. [16,17] and takes place according to reaction (1). In mammals this process is catalyzed by two different enzymes: choline dehydrogenase and betaine aldehyde dehydrogenase (AlDH), respectively [18]. Reaction (1) is analytically used for choline determination, but coupled to other reactions it is also the basis of the enzymatic determination of compounds containing choline such as PAF (platelet activating factor), phosphocholine, acetylcholine, phosphatidylcholine (PC), lyso-PC or sphingomyelin, which have important roles in structural and physiological human activity. For this purpose, reaction (1) is usually coupled to a second reaction in which the hydrogen peroxide participates in a later reaction (usually enzymatic) involving a fluorophore or a chromophore [19–21]. In addition to Ch, BA is also physiologically important [22] because it protects plants from environmental stress [23] and it has several functions in mammals (for example, as a source of methyl groups) [24].

In this paper we carry out a fundamental study on the analytical possibilities of FAD fluorescence of choline oxidase (ChOx). On the one hand, several specific behaviors of the fluorescence of this enzyme have been observed; in addition, the fluorescence signals permit the quantitative determination of Ch and a semi-quantitative estimation of BA concentration in mixtures. The FAD fluorescence gives more information than that obtained when the intrinsic UV fluorescence of ChOx is used [25].

2. Experimental

2.1. Apparatus

Steady state fluorescence measurements and contour plot (3D) spectral measurements were carried out with a Photon Technology International (PTI) Time Master fluorescence spectrometer (TM-2/2003). For lifetime measurements this instrument has a N_2 laser (GL-3300) that pumps a dye laser (plugged in to a frequency doubler for working with lower wavelengths) as a radiation source and a stroboscopic system as the detector. 3D spectral measurements were carried out with a Perkin-Elmer LS 50B luminometer. 3.5 mL Hellma quartz cuvettes with 10 mm path length were used. Different bandwidths were chosen in both excitation and emission monochromators.

Absorption measurements were carried out with an Agilent 8453 UV–visible spectrophotometer. This instrument uses a photodiode array (PDA) for simultaneous measurement of the complete UV–visible spectrum with a 1 nm slit.

An optical Foxy-R Oxygen Sensor (Ocean Optics), which is based on the O_2 collisional quenching of fluorescence, was used for monitoring dissolved oxygen during the enzymatic reaction. Optical fibers carried both the excitation light produced by the blue LED (450 nm) and the fluorescence generated to the detector.

2.2. Reagents

pH 8.5–10 0.1 M carbonate solutions were prepared from solid $NaHCO_3$ and Na_2CO_3, and pH 6–9 0.1 M phosphate solutions from solid Na_2HPO_4 and NaH_2PO_4. Choline oxidase from *Alcaligenes sp.* (ChOx) and from *Arthrobacter globiformis* ($ChOx_{Arg}$) (EC 1.1.3.17) were purchased from Sigma-Aldrich C5896 as lyophilized solids with 12–15 U mg^{-1} activity.

Choline chloride and betaine aldehyde chloride stock solutions were supplied by Sigma (C-1879 and B3650).

2.3. Analytical procedures, software and data processing

For lifetime measurements, the cuvette was filled with ChOx solution (10 U mL^{-1}) and the decay curves were obtained at pH 9 (λ_{exc}=410 nm; λ_{em}=520 nm) and pH 6 (λ_{exc}=450 nm; λ_{em}=520 nm). To obtain the FAD lifetime, the curves were fitted to an exponential function; the best fit was determined when $0.9 < \chi^2 < 1.2$.

For measurements in batch (λ_{exc}=410 nm; λ_{em}=520 nm), 1 mL ChOx solution and 1 mL pH 9 carbonate solution were added to the quartz cuvette (ChOx 2 U mL^{-1}). After a stabilization time of 120 min (this time could be suppressed or reduced but it is important to accurately measure the F_0 value), 40 μL of the analyte solution (Ch o BA stock solutions or sample solution) were added to the cuvette, the solution was shaken during 5 (± 1) s (aspirating and expelling with the same micropipette used for sample addition) and the fluorescence intensity was followed over the time.

A set of 25 synthetic mixtures of Choline (Ch) and Betaine Aldehyde (BA) was prepared with 5 levels of concentration for each analyte (BA: $5.35*10^{-6}$ M, $9.62*10^{-6}$ M, $1.92*10^{-5}$ M, $3.85*10^{-5}$ M, $5.13*10^{-5}$ M; Ch: $5.07*10^{-6}$ M, $9.73*10^{-6}$ M, $1.95*10^{-5}$ M, $4.05*10^{-5}$ M, $5.67*10^{-5}$ M) (see Electronic Supplementary Material, ESM, Fig. S1) taking 18 samples for calibration and leaving out 7 for validation. The fluorescence vs. time profiles, $F=f(t)$, for each mixture was followed according to the aforementioned procedure with enzyme concentration [ChOx]= 2.0 U mL^{-1}, at pH=9 and λ_{ex}=410 nm and λ_{em}=520 nm. Data were collected in Excel and then exported to the software UNSCRAMBLER v. 7.5 (Camo A/S, Trondheim, Norway) for normalization (every fluorescence value was divided by the initial F value, F_0), for data pretreatment and for univariate and multivariate analysis. The chemometric procedure of calibration included a test for outliers detection based on the comparison of variances of results; the rejected values were removed and were not included in the set. Finally, 16 samples were used for calibration and 5 for validation (Fig. S1).

For univariate calibration, and after normalizing the experimental data, the $F_{norm}=F/F_0=f(t)$ profiles were obtained; then the values H_{max} and A_t obtained from them (see ahead) (using the software Origin™) were used to build up the univariate calibration lines for Ch and BA.

For multivariate calibration, and after normalization, the data were autoscaled [26]. Autoscaling supposes centering and scaling the data to avoid undesirable fluctuations during the handling of data in order to get a better interpretation of the prediction model. The leave-one-out full cross-validation procedure was used to assess the robustness of the PLS constructed models. To find the optimal number of factors or latent variables (LVs) to be used with the PLS model a previously established method was used [27]. That is, the lowest number of LVs for which the validation (cross-validation) variance value does not differ significantly from the minimum, according to an F-test with probability P=0.25, was chosen.