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Analytical applications of the optical properties of ferric hemoglobin: A theoretical and experimental study

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ABSTRACT

Like other hemeproteins, ferric hemoglobin (HbIII) not only can act as a catalyzer in the oxidation of substrates by H_2O_2 but also can act alone as a H_2O_2 reducer. In this paper, the kinetic mechanism of the reaction between HbIII and H_2O_2 is studied, and a mathematical model is developed, which permits the determination of the kinetic constants from the absorbance measurement; the results indicate that each HbIII molecule is able to oxidize up to 5 H_2O_2 molecules without external regeneration. Second, it is demonstrated that the optical properties of HbIII can also be used as an indicator of this oxidation reaction, and analytical methods can be developed using this approach. A method for H_2O_2 peroxide determination is presented, which permits the analyte to be determined in a concentration range that depends on the HbIII concentration used and the measurement time, with an RSD better than 5%. In addition, methods for peracetic acid (PPA), the simultaneous determination of PAA + H_2O_2 and the use of HbIII/H_2O_2 as an indicator for oxidase enzymatic reactions (glucose/glucose oxidase) are outlined.

1. Introduction

The development of analytical methods based on the use of enzymatic reactions attracts considerable interest, owing to their specificity and reversibility. Within these analytical methods, several research groups have focused on the use of the spectroscopic properties of proteins [1–5], mainly fluorescence. However, as far as we know, the autoindicating fluorescence properties can only be used in those enzymes having flavin groups as coenzymes. Although many interesting analytes are involved in reactions catalyzed by these kind of enzymes (glucose, cholesterol, or choline), there are still many other compounds for which such reactions are not available. In these cases, researchers have been designed alternatives methodologies based on the use of the molecular absorption properties of proteins, mainly hemeproteins, because they are able of self-detecting hydrogen peroxide produced in a previous reaction.

The hemoproteins, present the hemo group as a cofactor, consist of a porphyrin ring linked to Fe atoms through central nitrogen. This hemo group has an intense absorption band in the UV region (Soret band or γ) and weak absorption bands in the visible and in the near IR region [6–9]. These absorption bands are highly dependent on the kind of hemo group and its axial ligands. Moreover, these optical properties also depend on the oxidation state of the hemo group and can consequently be used for the monitoring of the enzymatic reaction in which the hemoprotein takes part.

In previous work [10,11], we developed an analytical methodology that makes it possible to design and to optimize an autoindicating biosensor using hemoproteins both as bio-receptors and transducers. This methodology is based on the molecular absorption properties of HRP and its variations during the enzymatic reaction with H_2O_2.

In this work, ferric hemoglobin (HbIII) was selected from among the different hemoproteins due to its function as a transport protein and because it is able to react with oxygen peroxide through a pseudo-peroxidase catalytic cycle with reaction intermediates similar to that of HRP (peroxidase). However, there are differences between HbIII and HRP, which confirm that the autoindicating methodology could be valid for any hemoprotein. Like all heme proteins, HbIII is made up of an active redox heme group (in this case, ferrous) and its amino acidic surround. During the enzymatic reaction of Hb with H_2O_2, the reaction intermediates (compound I and compound II) and the native HbIII show different molecular absorption spectra. It is therefore possible to follow the enzymatic reaction through the changes in its molecular absorption spectrum owing to the different reaction intermediates that are proportional to H_2O_2 concentration.

A mathematical model relating the HbIII absorbance variation during the reaction with the H_2O_2 concentration has been developed to provide theoretical support for the method and to predict its application to other compounds. The kinetic of the HbIII with H_2O_2 has also been clarified. This paper also provides new information about the HbIII

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cycle, the redox behavior of heme proteins and spectral information about reaction intermediates.

2. Experimental

2.1. Material and methods

2.1.1. Apparatus

The molecular absorption measurements were carried out with a Hewlett-Packard 8452A diode-array spectrometer and a Perkin-Elmer Lambda 5 (spectral bandwidth 2 nm) spectrometer. A quartz cuvette with a 1-cm optical pathway was used.

2.1.2. Reagents

The buffer solution was a 0.1-M phosphate solution of pH 6 (from solid KH_2PO_4 and solid Na_2HPO_4). Ferric human hemoglobin and ferrous human hemoglobin were supplied by Sigma (Sigma H-7379 and Sigma H-0267 respectively). Glucose oxidase was taken from *Aspergillus niger*, and EC 1.1.3.4 (Sigma G-7141) was of 179000 IU g^{-1} of lyophilized solid. Hydrogen peroxide solutions were prepared from dilution of the commercial solution (Panreac, 30% (w/v)). The hydrogen peroxide concentration was determined by redox titration with permanganate. Peracetic acid solutions were prepared from dilution of the commercial solution (Fluka 77240 39% d = 1.15 g mL^{-1}). Glucose solutions were prepared from solid β-D-glucose (Sigma G-5250) in buffer solution (the solution was left for two hours to achieve the equilibrium between β-D-glucose and α-D-glucose). Triton X-100 from Sigma was also used to stabilize the hemoglobin solutions.

3. Theoretical study of the process: mechanism of the HbIII/H_2O_2 reaction

3.1. Kinetic pathways

As occurs with other hemeproteins, the reaction mechanism between ferric hemoglobin (HbIII) and hydrogen peroxide is complex in the absence of an external reducer (substrate). The whole process includes several steps and combines several alternative pathways but can be summarized as follows (Fig. 1) [12–15]:

1. Just after mixing HbIII and H_2O_2, the heme group is oxidized to an unstable compound (compound I, having a formal +5 oxidation state and represented as HbV).
2. Compound I is not shown in Fig. 1 because it immediately decays to the intermediate compound II, with the heme group in a +4 oxidation state (HbIV). The mechanism driving this reduction is the so-called intramolecular reduction (IntraR) in which the heme group is reduced by its amino acid environment (tryptophan, tyrosine, or cysteine). As a result, this proteinic part of the hemoglobin becomes oxidized (this is represented in Fig. 1 by a superscript in the corresponding species indicating the formal oxidation state of the proteinic part).
3. Compound II with the proteinic part oxidized ($HbIV^+$) decays to the original ferric state following one of two alternatives pathways:
   a) by another IntraR to give $HbIII^{2+}$
   b) by the so-called intermolecular reduction process (InterR) in which $HbIV^+$ is reduced by the proteinic part of another hemoglobin molecule (HbIII), so both molecules give the same species: $HbIII^+$.
4. The ferric hemoglobin ($HbIII^+$, $HbIII^{2+}$) thus obtained is ready to react with another molecule of H_2O_2, and the process represented by steps 1 to 3 is repeated. This cycle can take place several times until the full oxidization of the amino acidic residues is achieved. However, two problems arise:
   * A fraction of the hemoglobin does not return to the original ferric state and remains inactive mainly as compound II (and partially as compound I).
   * The kinetic constants in each cycle become slower than in the previous cycle.
5. Finally, it is important to emphasize that at very high H_2O_2 concentrations the degradation of the hemoglobin takes place [16–18]. In this process, $HbIV^+$ reacts with the excess of H_2O_2 to give a superoxide intermediate (HbIII-$O_2^{\bullet-}$). The superoxide anion in the heme site

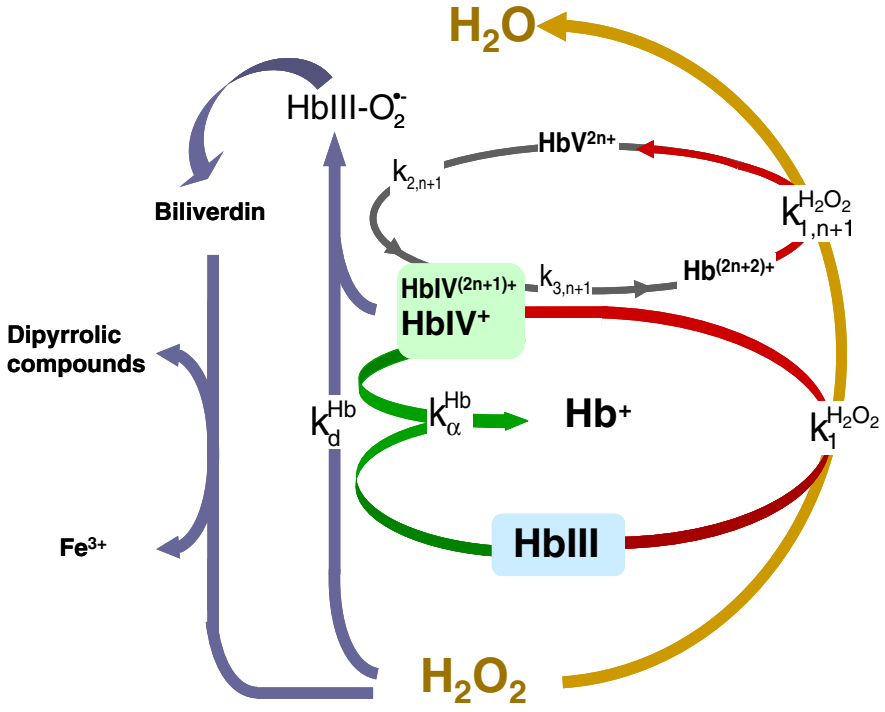

Fig. 1. Reaction mechanism of HbIII with H_2O_2.