CONFORMATIONAL CHANGES OF BOVINE HEMOGLOBIN AT DIFFERENT PH VALUES, STUDIED BY ATR FT-IR SPECTROSCOPY

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Abstract. The conformational changes of bovine hemoglobin at different pH values have been studied by FT-IR spectrometry using an Attenuated Total Reflectance (ATR) accessory. Changes in the amide bands in Fourier transform infrared spectra of proteins are generally attributed to alterations in protein secondary structure. In this study the spectra of hemoglobin at different pH values were analyzed. The second-derivative analysis of infrared spectra permits direct quantitative analysis of the secondary structural components of proteins by integration and curve fitting

Key words: ATR-FTIR, proteins, secondary structure.

INTRODUCTION

Secondary structure of proteins refers to the organization of amino acid residues in a polypeptide chain and is predominantly composed of α-helical and β-sheet sequences [6, 7]. There are several experimental techniques for protein secondary structure determination such as circular dichroism (CD), Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR), Raman spectroscopy, and X-ray diffraction. The majority of three-dimensional coordinates currently available in the protein data bank (PDB) were obtained from NMR or X-ray diffraction spectroscopy. Therefore, the techniques of NMR and X-ray diffraction are used, mainly to determine the coordinates of three-dimensional. Circular dichroism, Raman spectroscopy, and FT-IR spectroscopy are routinely used to rapidly classify the status or alteration of proteins according to secondary structure. The alteration of the secondary structure of proteins refer to changes in ratio among three common structures, namely alpha helices, beta sheets, and turns. That which cannot be classified as one of the standard three classes is usually grouped into a category called "other", "random coil" or "aggregates".

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In this paper conformational changes of bovine hemoglobin, due to modification of pH have been studied by FT-IR spectroscopy [3, 1]. Structural information of lyophilized hemoglobin was obtained by analysis of the conformational-sensitive amide I band using Attenuated Total Reflectance FT-IR spectroscopy (ATR-FTIR). The second derivative spectrum was performed in order to overcome the bands overlapping due to the different C=O stretching vibrations of each type of secondary structure (i.e. α-helix, β-sheet, turns, unordered) [9, 10].

MATERIALS AND METHODS

MATERIALS AND SAMPLE PREPARATIONS

Powdered bovine hemoglobin (> 95% methemoglobin) from SIGMA Chemicals, were used without further purification. Proteins were hydrated in phosphate buffer physiological saline at a final concentration of 10^{-3} M. The pH values were adjusted to the desired value in the range 2.5–9.5.

INFRARED SPECTROSCOPY

The FT-IR spectra of proteins were recorded in the region 4000–800 cm^{-1} by a Bruker EQUINOX 55 spectrometer, using an Attenuated Total Reflectance accessory with a scanning speed of 32 cm^{-1} min^{-1} with the spectral width 2.0 cm^{-1}. The internal reflection element was a ZnSe ATR plate (50 x 20 x 2 mm) with an aperture angle of 45°. A total of 128 scans were accumulated for each spectrum. Spectra were recorded at a nominal resolution of 2 cm^{-1}.

METHODS

In the Fourier domain derivative, the second-derivative analysis of infrared spectra permits direct quantitative analysis of the secondary structural components of proteins [4]. The basic idea of this method, is that, the intrinsic shape of an infrared band absorbance of spectrum $A(\nu)$ is approximated by a Lorentzian function:

$$A(\nu) = \frac{A_0 \Gamma^2}{\Gamma^2 + (\nu - \nu_0)^2} \tag{1}$$

in which ν is the wavenumber (in units of cm^{-1}), A_0 is maximum absorbance of the band corresponding to wavenumber ν_0 and Γ is half-width at half-height of original spectrum. The Fourier transform of of infrared band spectrum is given by:

$$I(x) = F\{A(\nu)\} = \int_0^\infty A(\nu)\cos(2\pi\nu\, x)d\nu = \frac{1}{2}A_0\Gamma\cos(2\pi\nu_0\, x)\exp(-2\pi\Gamma\, x) \quad (2)$$

where x is the frequency (in units of cm). Between spectrum and its Fourier transform $I(x)$ there is the following relation [5]:

$$A(\nu) = F\{I(\nu)\} = \int_0^\infty I(\nu)\cos(2\pi\nu\, x)dx \quad (3)$$

The second order derivative is given by:

$$\frac{d^2A(\nu)}{d\nu^2} = \int_0^\infty (-2\pi x)^2 I(x)\cos(2\pi\nu\, x)dx \quad (4)$$

Substituting (2) in (4), the second derivative band spectrum becomes:

$$A^{II} = \frac{d^2A}{d\nu^2} = -\frac{1}{\pi\Gamma^{II}}\frac{2a\left(1-3a\nu^2\right)}{\left[1+a\nu^2\right]^3} \quad (5)$$

in which $a = \frac{1}{\left(\Gamma^{II}\right)^2}$, with Γ^{II} the half-width at half-height of the second derivative spectrum. The half-width (Γ^{II}) and the maximum absorbance of the band corresponding to wavenumber ν_0 (A_0^{II}) of the second derivative band spectrum are related with original line intensity by relations $\frac{\Gamma}{\Gamma^{II}} = 2.7$ and $A_0^{II} = -\frac{2A_0}{\Gamma^2}$ respectively [2]. By this procedure, the signal to noise ratio of the spectrum is minimized and is amplified the disproportionalety in the weak features of the spectrum. Thus, the areas corresponding to the different types of secondary structure are quantitatively and qualitatively evaluated by integration and curve fitting.

RESULTS AND DISCUSSIONS

The best information from infrared protein spectra is obtained in the amide I band, which appears between 1700 and 1600 cm^{-1}. These band arises mainly from C=O group stretching vibration. On the other hand, each type of secondary structure gives rise to different C=O stretching frequencies resulting in characteristic band positions [8].

The second derivatives of all spectra were calculated using Origin 6.0 program. Before starting the fitting procedure, the obtained depths of the minima in the second derivative spectrum and, subsequently, the calculated maximum intensities were corrected for the interference of all neighboring peaks. The curve fitting is performed by stepwise iterative adjustment towards a minimum root-mean-square error of the different parameters determining the shape and position of the absorption peaks (Fig. 1).

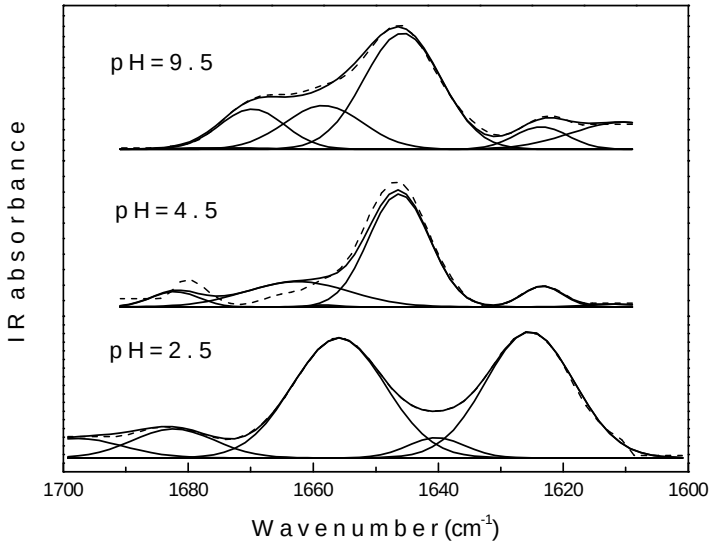

Fig. 1. Curve-fitted inverted second-derivative amide I spectra of lyophilized hemoglobine.

Our results on relative areas and assignments of secondary structure of hemoglobin at different pH are presented in Table 1 and Table 2.

Table 1

Relative areas and assignments of infrared second derivative amide I band oflyophilized bovine hemoglobin at pH 2.5, 3.5 and 4.5

pH = 2.5		pH = 3.5		pH = 4.5		
$\nu(cm^{-1})$	Areas(%)	$\nu(cm^{-1})$	Areas (%)	$\nu(cm^{-1})$	Areas(%)	Assignments
1698	6.6	1698	3.1	1698	7.2	Aggregates
1682	8.5	1682	21.7	1682	7.0	Turns
1656	40.4	1656	36.9	1654	61.6	α-helix
1640	4.1	1640	3.9	1640	–	Random
1625	40.4	1625	34.4	1626	23.2	β-sheets
1620	–	1620	–	1620	1.0	Aggregates

Table 2

Relative areas and assignments of infrared second derivative amide I band oflyophilized bovine hemoglobin at pH 6.7, 8.1 and 9.5

pH = 6.7		pH = 8.1		pH = 9.5		
ν(cm^{-1})	Areas(%)	ν(cm^{-1})	Areas(%)	ν(cm^{-1})	Areas(%)	Assignments
1698	14.6	1698	17.1	1698	14.7	Aggregates
1681	6.6	1682	4.1	1682	6.2	Turns
1656	42.8	1654	48.1	1655	46.8	α-helix
1640	22.8	1640	18.1	1640	17.8	Random
1630	5.5	1626	11.6	1626	13.6	β-sheets
1620	7.7	1620	1.0	1620	0.9	Aggregates

The secondary structure of the proteins is very sensitive to the environment in which the proteins are kept. As expected the pH of the environment has a dramatical impact upon protein's secondary structure. The results of qualitative and quantitative analysis [11] by curve fitting to the inverted second derivative spectra of amide I features of lyophilized hemoglobin reveals a decrease in β-sheet content as the pH values increases and a decrease in α-helix content at lower or higher pH values as well as a decrease in turns content. The maximum value of the α-helix content was found at pH = 4.8 (Fig. 2). On the other hand, at higher pH values we observed an increase in random coils content, concerning the aggregation of protein, at neutral pH value we observed a maximum value of its content.

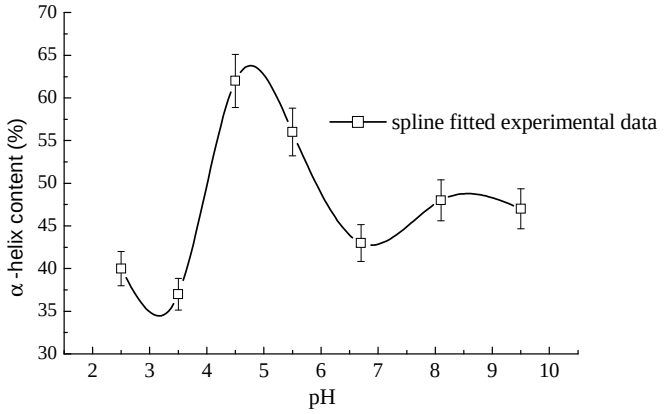

Fig. 2. Variation of the α–helix content with pH values.

CONCLUSIONS

The results presented in this article demonstrate the utility of the ATR-FTIR spectroscopic technique for characterisation of protein secondary structures in the processes, which involve their denaturation. Simultaneous qualitative and quantitative analysis of amide I ATR-FTIR spectra of hemoglobin by curve fitting of the inverted second derivative spectra reveals changes in α-helix content of hemoglobin with a maximum in at pH ≈ 5.

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