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ЦЕНТРАЛЬНЫЙ НАУЧНО-ИССЛЕДОВАТЕЛЬСКИЙ ИНСТИТУТ
ИНФОРМАЦИИ И ТЕХНИКО-ЭКОНОМИЧЕСКИХ ИССЛЕДОВАНИЙ
ПО АТОМНОЙ НАУКЕ И ТЕХНИКЕ

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ESR SPECTRA OF Cu(II) - BSA COMPLEXES VERSUS pH
(Part I)

ЕРЕВАН-1984

Introduction

The synchrotron radiation (SR) arising in electron accelerators during the motion of electrons accelerated in magnetic field is widely applied in different fields [1-3]. Of special interest are the biological investigations accomplished using SR by means of EXAFS (extended X-ray absorption fine structure) method [4-14] which is theoretically developed in [15-22]. This method has been successfully applied in measuring the interatomic distances in rubredoxin protein [10-11], in the investigation of the hemoglobin conformational changes at its oxygenation [6]. The basics of EXAFS method are described in detail in [23], and the recent achievements due to the application of this method to biological molecules are systemized in [24]. Though this method is very promising for the investigation of the structure of metal-containing proteins and enzymes, considerable difficulties arise when using it in the identification of ligand atoms surrounding the central ion of metal. The ESR method is free of this shortcoming. Using this method one may by means of the superhyperfine structure of ESR spectra, carry out an unambiguous identification of ligand atoms, the di-

tance between the central atom of metal and its ligands remaining unknown [25]. Thus these two methods may be considered as kind of mutually complementary, if the central atom of metal is paramagnetic. In the case when it is diamagnetic, one should replace it with a paramagnetic ion, though for the EXAFS method this limitation is not essential.

In connection with the above stated, we have felt it expedient to choose a problem to which we might apply both ESR and EXAFS methods. At the first stage of investigation we have used the ESR method only, though the use of the EXAFS method, supplementing the data obtained by the ESR method, would have been desirable.

The essence of the chosen problem is the following. Back in 1948 Klotz et al. [26] have begun to investigate the interaction between Cu(II) and bovine serum albumin in connection with the clarification of the role of Cu(II) in the mechanism of the transporting function of this protein. The further investigation of this problem was carried out in papers systemized in reviews and monographs [27-37]. The results obtained in these papers, carried out in the main by spectrophotometric methods, potentiometric titraton, proton displacement reaction, equilibrium dialysis etc, were reduced to the fact that at the complex formation of Cu(II) with BSA two basic binding sites are formed. One of them possesses optical absorption at 650mμ wave length and is labile, the other, having an absorption peak at 525mμ, is stable, and both these sites behave differently depending on pH. A large number of investigations on this problem are carried out by the

above methods, and in none of them the ESR method is used.

The purpose of our paper is to investigate by the ESR method the binding sites in Cu(II)-BSA versus pH. At the same time the paper is aimed at showing the possibility of applying the EXAFS method to this problem to measure the distance between Cu(II) and ligands surrounding it at various pH values for appropriate binding sites.

The clarification of structural parameters of binding sites, which may be accomplished by the EXAFS method, will allow to come closer to the understanding of the role of Cu(II) in the mechanism of the BSA transporting function in the process of organism's activity. Since copper is a part of the active site of most metal containing enzymes, then the developed approach to the investigation of the above special problem may be used in the case of copper containing enzymes.

The paper is composed of two parts. In the first the ESR spectra of Cu(II)-BSA complexes are presented, and the behavior of appropriate binding sites versus pH is investigated. In the second part the identification of the complexes formed is carried out and an attempt is made to predict some data of EXAFS spectra basing on experimental results obtained by the ESR method.

A brief report of this paper was recently published in the form of theses [38]. Some aspects of this problem will be published in subsequent reports.

Material and Methods

A lyophilized preparation of bovine serum albumin of the Olayna (Latvian SSR, USSR) plant of chemical reagents was used. The salt $CuCl_2 \cdot 2H_2O$ was used. The Cu(II)-BSA complexes were formed by the technique described in [39]. The BSA concentration was 0.6 mmol/l, that of Cu(II) 2.4 mmol/l, the ionic strength was 0.16 mol/l. The ESR spectra of Cu(II)-BSA were recorded on the Varian E-4 ESR spectrometer approximately at -190°C at 10 mW power of the klystron and 1.0 mT amplitude of high frequency magnetic modulation. The measurement of the pH value was carried out by means of the pH-673 pH-meter. The calibration of pH-meter was carried out by standard buffer solutions. The measurement error was pH = $\pm$ 0.025. The required pH values were obtained by adding $NaOH$ into the initial solution. Buffer solutions were not used in order to avoid the formation of Cu(II)-BSA-buffer complexes [27].

Results and Discussion

In fig.1 the ESR spectra of Cu(II)-BSA versus pH are presented. Consider first the changes of these spectra in the range of small values of magnetic field: from 260.0 to 300.0 mT. For convenience we have enumerated some observable peaks. At pH 6.55, in the main, peaks 1 and 2 are observed. With the pH increase two more additional peaks 3 and 4 emerge, whose intensity increases with pH, whereas that of peaks 1 and 2 decreases. At pH 9.30 in the spectrum, in the main, peaks 3 and 4 are present. Such changes of the observed ESR spectra form may be

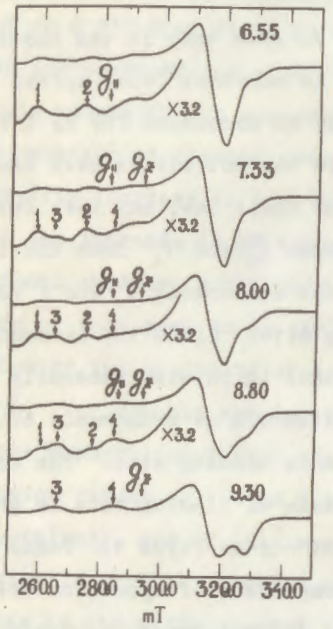


Fig.1

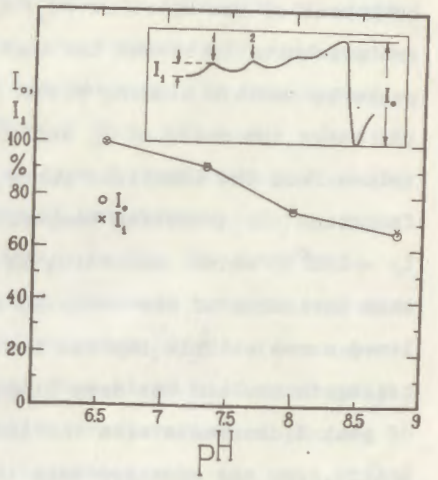


Fig.2

Relative dependence of the components I_o and I_1 of ESR spectra of Cu(II)-BSA in 0.16 mol/l KCl as a function of pH.

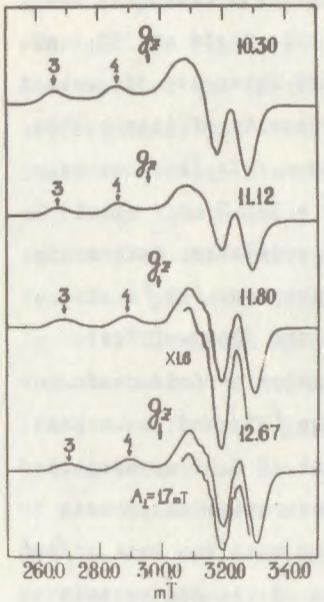
ESR spectra of Cu(II)-BSA at - 190°C in 0.16 mol/l KCl as a function of pH (figures in the right corner are for the pH value; figures with arrows on the left are for components of hyperfine structure of appropriate binding sites; values of $g_{\parallel}$ and g_2 for each binding site are shown with arrows; figures 3.2 indicate the amplification multiplicity of additionally recorded spectra at pH 6.55, 7.33, 8.00 and 8.80).

accounted for by the presence in the sample of two types of bound complexes which have a different concentration at different pH. In fig.2, in a relative scale, values of the central component of the spectrum (I_o) and the profile (I_1) versus pH are presented to reveal the correspondence of the observed peaks to certain binding sites. It is seen that in the observed pH range the ratio of I_o and I_1 is constant (some spread of values from the idealized curve may be accounted for by the fact that the detection temperature has not always been exactly - 190°C, which, according to the Curie law, may have led to some deviation of the observed values I_o and I_1 from the idealized curve). This implies that the components I_o and I_1 should belong to one and the same binding site. Since the intensity of peak 2 decreases with the increase in pH simultaneously with peak 1, one may consider that they should be components of hyperfine structure of one and the same binding site. The approximate form of this type ESR spectrum of binding site is presented in fig. 2 in the upper right corner (type 1). Peaks 3 and 4 should then correspond to components of hyperfine splitting of another binding site whose ESR spectrum's approximate form in a "pure" form may be presented in fig.1 at pH 9.30 (type 2): Let us turn to the consideration of the ESR spectrum in fig. 1 in the range of large values of magnetic fields: from 320.0 to 340.0 mT. At pH 6.55 in the magnetic field of approximately 330.0 mT a component is observed, whose intensity increases with pH and at pH 9.3 is observed in an explicit form. This component cannot be assigned to a part of a spectrum of type 1 complex, since with the increase in pH, when the relative

intensity of peaks 1 and 2 of type complexes decreases, the intensity of this component in the magnetic field of 330.0 mT increases. At pH 9.30, when the relative intensity of peaks 1 and 2 is practically near zero, the intensity of this component in a 330.0 mT field is at its maximum. In this connection the observed spectrum component in a 330.0 mT field should belong to the spectrum of type 2 complexes, indicating the presence of rhombic symmetry of environment [25]. The intensity increase of this component in the 330.0 mT field with the increase in pH apparently indicates protein conformational changes in the observed pH range [37] and the appearance of this peak in an explicit form at pH 9.30 apparently indicates the beginning of alkaline denaturation of protein [25]. Our results are in good agreement with the data of [40] on the absorption of 8.87 MHz ultrasound by the BSA protein solution versus pH (at pH 9 the minimum absorption coefficient is obtained), and of [41] where an abrupt increase in the intensity of the ESR spectrum central line and an antibatic decrease in its width at pH 9 is shown by means of a spin-labelled BSA.

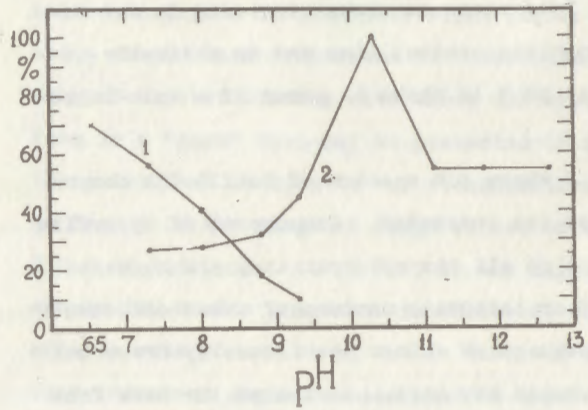
Consider now fig.3 where ESR spectra of Cu(II)-BSA compexes at higher pH values are presented. Components of hyperfine splitting are distinct on all the spectra: they are numbered 3 and 4. At pH 10.30 an intensity maximum of these components is observed. At subsequent pH values the intensity drops and practically doesn't change any more. At the pH increase from 11.1 to 11.8 there occurs a substantial change in spectra: the components of hyperfine structure noticeably broaden, an essen-

Fig.3



ESR spectra of Cu(II)-BSA at -190°C in 0.16 mol/l KCl as a function of pH (figures in the right corner are for pH values; 3 and 4 are for the components of hyperfine structure from one binding site; values of g_z from this binding site are shown with an arrow; figure 1.6 indicates the amplification multiplicity of additionally recorded part of the ESR spectrum at pH 11.80; at pH 12.67 the additional recording of the part of the spectrum is carried out at a smaller velocity of passage and the same amplification coefficient).

Fig.4



Dependence of relative intensity of components of hyperfine structure for type 1 (curve 1) and type 2 (curve 2) complexes on pH.

tial decrease in g_z is observed, and the form of spectra changes substantially in the range of magnetic field values from 310.0 mT to 330.0 mT. Similar changes in BSA solutions are described also in [40] according to which a substantial absorption of ultrasound is observed in the pH range 11.0 to 11.8. The further pH increase up to 12.67, as is seen from fig.3, does not lead to substantial changes of the ESR spectrum form.

Consider in more detail the changes of the form of spectra in fig.3 in the range of magnetic field values from 310.0 to 330.0 mT. At pH 10.3 one may notice weak superposed tracks of super-hyperfine structure components on the ESR spectrum basic component in the range of g_y. A similar tendency is observed also in the case of pH 11.12. At pH 11.80, however, there occur abrupt changes of spectrum forms; in the range of g_y there appear distinct components of super-hyperfine structure, which in the spectrum at pH 11.80 are written down also at an amplification 1.6 times higher. At the maximum pH value 12.67 these components are reproduced. At this fact indicates also the additional recording of the spectrum at a smaller velocity of passage and the same coefficient of amplification of ESR spectrometer. Note that the appearance of distinct components of super-hyperfine structure takes place simultaneously with the decrease in g_z from pH 11.12 to pH 11.80, which is an evidence [25] of the ligand atoms coming closer to the central Cu(II) atom in the given binding center (type 2). It follows from the spectrum at pH 12.67 that the number of observed components of superhyperfine structure is nine with a 1,7 mT splitting. The question of using these data for the identifi-

cation of type 2 complexes will be considered in the subsequent paper.

In fig.4 two curves versus pH are presented in a relative scale. Curve 1 corresponds to the change of relative intensity of the hyperfine structure component (peak 1 in fig.1) for type 1 complexes, and curve 2 of that for type 2 complexes (peak 3 in fig.1 and fig.3). With the pH increase curve 1 drops monotonically. If we extrapolate these data up to the X-axis, we may obtain that at pH being approximately 10 the relative intensity of peak 1 in the sample in fig.1, proportional to the concentration of type 1 complexes, is practically equal to zero. Namely at pH≃10, in accordance with [37] there occur in BSA irreversible conformational changes. This coincidence is apparently not accidental, and there is a certain relation between them. The decrease in the relative concentration of type 1 complexes with the increase in pH indicates at a type 1 complex weakly bound to protein. Besides, with the pH increase conformational changes in BSA are observed caused by redistribution of bonds between two disulfide bridges [37], which apparently supports the decrease in the concentration of type 1 complexes, especially in predenaturation pH range.

In contrast with curve 1, curve 2 with the increase in pH behaves differently: at $9 > pH \geq 8$ it begins to slowly increase, which is apparently related to the "turning round" of albumin molecules [37]. At $pH \geq 9$ there occurs a more abrupt increase in this curve, apparently due to a considerable increase in the negative charge on protein with pH. At pH 10.3 the curve reaches its maximum, then it drops and at the further pH increase remains practically constant.

It should be noted that at pH 8.4 an isobestic point is observed, where both types of binding centers should have an equal consentration in solution.

In fig.5 values of constants of hyperfine splitting for

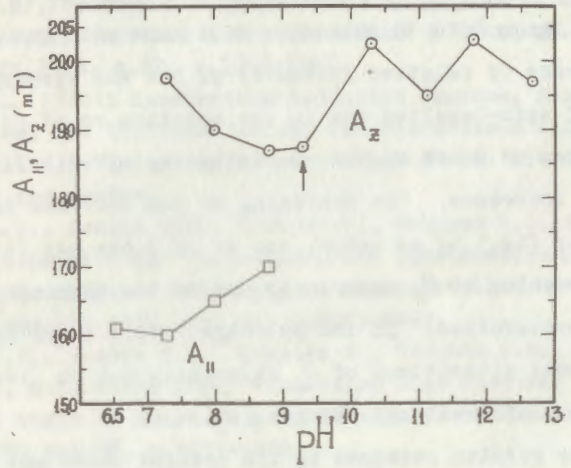


Fig.5

Dependence of the constant of hyperfine splitting for binding sites of type 1 (A_{II}) and type 2 ($A_{\perp}$) on pH.

type 1 (A_{II}) and type 2 ($A_{\perp}$) complexes are presented. One may see that the values of A_{II} are much smaller than $A_{\perp}$ in all the investigated pH range. With the pH increase A_{II} tends to increase its absolute value approximately by 1.0 mT, whereas $A_{\perp}$ at first monotonically decreases with the pH increase from 7.3 to 9.0 by 1.0 mT, and then increases up to pH 10.3. The form of this curve is similar to that of ultrasound absorption by the BSA protein solution at a frequency 8.87 MHz in the pH ran-

ge 7 to 10 [40], though the alterations of the constant A_2 observed by us are more substantial: from pH 7 on the curve of ultrasound absorption decreases with the minimum at pH 9, then it begins to slowly increase and at pH 10.3 the absorption value is larger than at pH 7.3. This dependence of A_2 on pH in the pH range 9 to 10 coincides well also with the curve of the dependence of relative intensity of the ESR spectra central line of spin-labelled BSA in the solution on pH [41]: beginning from pH 9 and higher the intensity of this line substantially increases. The beginning of the increase in A_2 is shown in fig.5 by an arrow, and at pH 9 one may find out the minimum value of A_2 apparently due to the beginning of protein denaturation. In the pH range > 10.3 there appear some periodical alterations of A_2 apparently due to irreversible protein conformational changes [37].

The results obtained in the present paper may be supplemented with structural data of EXAFS method using the synchrotron radiation of electron synchrotron. These problems will be considered in detail in the subsequent paper.

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Р.А.АСАТУРЯН

СПЕКТРЫ ЭПР КОМПЛЕКСОВ Cu(II)- БСА ОТ pH

(часть I)

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