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Binding of heavy metals with C-Phycocyanin: A comparison between equilibrium dialysis, fluorescence and absorption titration

Eteri Gelagutashvili

Andronikashvili Institute of Physics, Ivane Javakhishvili Tbilisi State University, Tbilisi, Georgia

Email address:
eterige@gmail.com, gelaguta@yahoo.com

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Abstract: The energetic of interaction of metal ions with C-Phycocyanin (C-PC) Spirulina platensis were investigated using optical and thermodynamic methods. Comparison of the binding constants of heavy metals with C-PC by the instrumentality of difference methods shown, that binding affinities of toxic and heavy metal ions for C-PC strongly depend upon the metal. The efficiency of interaction for different metals is arranged in the same sequence for the absorption titration as it is for fluorescence. Obtained results using equilibrium dialysis are in good agreement with results using fluorescence spectroscopy in the case Ag+, but difference is observed for Ni2+ and Cr3+ ions.

Keywords: C-Phycocyanin, Heavy Metals, Binding Constants

1. Introduction

Spirulina is a photosynthetic, filamentous, multicellular, and blue green microalgae [1]. Spirulina is a traditional food of some Mexican and African people. The bioactive protein of Spirulina platensis C-phycocyanin (C-PC) could be used in the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's deceases brought on by oxidative stress-induced neuronal injury [2]. An aqueous extract Spirulina platensis inhibited HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells (PBMC), and Langerhans cells [3]. Antibacterial activities of C-PC (Spirulina platensis) were obtained from various human pathogens [4]. Addition of heavy metals to intact cells of the cyanobacterium, Spirulina platensis caused alterations in the whole chain and photosystem II catalyzed electron transport activities [5, 6].

In spite of the fact, that there is significant increase in the interest on studying the biosorption of metals with microorganisms and their components, the mechanism of metal ions interaction with them is essentially unknown.

In this work the interaction of metal ions with C-phycocyanin (C-PC) Spirulina platensis were investigated using optical and thermodynamic methods.

2. Materials and Methods

C-PC was isolated from our laboratory culture of Spirulina platensis (strain IPPAS B-256) according to the Teale and Dale [7] method with some modifications. The extraction of C-PC was carried out in 0.1M Na, K phosphate buffer, pH 6.0. Initially the preparation was purified by the method of deposition in saturated (NH4)2SO4 solution. The final stage of purification was performed on a chromatography column filled with DEAE-cellulose, previously equilibrated in 50mM acetate buffer, pH 5.2. To determine the degree of purity of the samples, spectrophotometric (wavelenhths 250-750 nm) and electrophoretic methods were used. The concentration of C-PC was determined by ultraviolet-visible spectroscopy using a value of ε λ=615nm=279000 M-1cm-1 for the absorption coefficient. The purity of the protein was assessed from the ratio of absorbances at λ =615nm and λ=280 nm (A615/A280 ≥4).

Data analysis. Absorption titration was performed in the 620 nm. Fitting of absorption titration has been performed model, where two extinction coefficients εb and e ε f are used for a chromophore bound to C-PC and free in solution [6, 7]. The intrinsic binding constant K was determined

from the plot of D/Δ ε ap vs D, where D is the concentration of metal, Δ ε ap =[ε a- ε f] and Δ ε =[εb-εf][8,9]. The apparent extinction coefficient ε a, is obtained by calculating Aobs/[C-PC] , Aobs optical density, εb and εf correspond to the extinction coefficient of the bound form of C-PC and the extinction coefficient of free C-PC, respectively. The data were fitted to eq.1, with a slope equal to 1/Δ ε, and a Y –intercept equal to 1/ΔεK. εb was determined from Δε , and K was obtained from the ratio of the slope to the Y intercept.

$$1/\Delta \varepsilon ap = D/\Delta \varepsilon ap + 1/\Delta \varepsilon K \quad (1)$$

The interaction of heavy and toxic metal ions with C-PC has been studied by laser fluorescence spectroscopy. The intensity of C-PC fluorescence, observed in the range 580-690 nm. It should be noted that at fluorescence titration the number of free metal ions is determined by indirect method [10]. In this case the concentration of the free probe was determined using eq.2, where CT is the concentration of the probe added, CF is the concentration of the free probe, and I and I0 are the fluorescence intensities in the presence and in the absence of metal ion, respectively. P is the ratio of the observed fluorescence quantum yield of the bound probe to that of the probe.

$$CF = CT\ (I0/I-P)\ (1-P) \quad (2)$$

The value of P was obtained from a plot of I0/ I vs 1/[C-PC] such that it is the limiting fluorescence yield given by the Y-intercept. The amount of bound probe CB at the concentration was equal to

CT- CF. metal binding to C-PC was analyzed by the Scatchard [11] and Hill[12] graphical methods with certain mathematical considerations developed later[13].

Scatchard,s basic binding model derives from a simple consideration of the law of mass action K=[complex]/[free ion][free binding sites]. If we have N classes of independent sites, each class i having ni sites each with the same binding constant ki (with ∑ki=K),then

$$n = \lim_{\log[m]\to\infty} r\ ,\ r = \sum_{i=1}^{N} \frac{k_i n_i m}{1 + k_i m} \quad (3)$$

where r is the moles of bound metal ions per mole of protein, m, the concentration of free metal ions. From the view point of the experimenter,

$$r = Cbound/[C\text{-}PC] \quad (4)$$

where Cbound is the concentration of C-PC. Nonlinearity of Scatchard binding plots (Y vs logm, where Y=r/n) may arise from several effects (n- number of binding sites). At Y=0.5, the binding constant is estimated from

–logm=logK. The χ2 distribution was used as the criterion of goodness of fit.

The Hill equation is

$$(1/nH)\log Y/1-Y = \log K + \log m, \quad (5)$$

where nH is the Hill coefficient (an index of cooperativity).

The binding constant K was used to calculate the Gibbs free energy from the standard relation ΔG0=-RTlnK, (6) where R is the gas constant, T is the absolute temperature and ΔG0 the Gibbs free energy.

Equilibrium dialysis experiments were performed in a two-chambered Plexiglass apparatus. The chamber capacity was 5 ml. The (visking) membrane separating the chambers was 30 μm thick. The initial metal concentration was varied within the range 10-6-10-4 M. During equilibrium dialysis, protein solution (C-PC) was placed in one chambers and metal solution in the other. The semi-permeable membrane separating the chambers is impenetrable only for protein. After attaining equilibrium, some of the metal ions remain free and the remainder are bound to C-PC, i.e. [metal] total=[metal]free+[metal-C-PC]. The C-PC concentration was 10-4M. Samples were analyzed for metal by flame atomic absorption spectrometry (wavelengths ((Ag) =328.1nm, (Cr) =357.9nm, (Pb)=283.3nm, (Cd)=228nm, (Cu)=324.75nm,(Ni)= 232nm. All the experiments were carried out at 230C, at various ionic strength (50mM, 20mM, 2 mM).

Table 1. *Binding affinity of metal ions to C-PC*

Element	Binding constant K x104 M-1	Gibbs free energy - ΔG0 Kcal/mol	Correlation Coefficient R
Mg2+	0.11	4.14	0.98
Mn2+	0.15	4.32	0.96
Al3+	0.14	4.28	0.91
Co2+	0.21	4.52	0.97
Ni2+	0.23	4.57	0.95
Zn2+	0.19	4.46	0.97
Cd2+	0.28	4.69	0.95
Ag+	19.49	7.2	0.95
Hg2+	107.15	8.2	0.99
Pb2+	2.19	5.9	0.94
Sr2+	0.14	4.32	0.96
Cr3+	0.39	4.9	0.96
Cu2+	0.34	4.8	0.93

3. Results and Discussions

3.1. Absorption Titration

The electron absorption spectra of C-PC in the presence of increasing amounts of the metal ions showed strong reduction of the peak intensities (hypochromicity). The absorbance variation at λ = 620 nm with increase of metal ions concentrations was used to construct the half-reciprocal plot. The binding constants were estimated from these plots. In Table 1 are presented the binding constants K and the Gibbs free energy ΔG0 for metal-C-PC complexes. It is evident, that binding affinities of metal ions to C-PC strongly depend upon the metal and the binding constants are arranged in the descending order as follows:

Hg(II) > Ag(I) > Pb(II) > Cr(III) > Cu(II) > Cd(II) > Ni(II) > Co(II) > Zn(II)> Mn(II) > Sr(II) > Al(III) > Mg(II).

The results of the study indicate (Table 1) that calculated values of ΔG0 are characteristic for hydrogen bonds of Pb2+, Cu2+, Cd2+,, Co2+, Ni2+, Zn2+, Cr3+, Mg2+, Mn2+, Al3+, Sr2+, (4.14÷5.9 kcal/mol), whereas Ag+, is almost on the verge (7.2 kcal/mol) and Hg2+ exceeds the energy of hydrogen bonding (8.2 kcal/mol). It is clear, that when using half-reciprocal plot, linear fits for determination of binding constants always give high correlation coefficient value for these complexes (> 0.90).

3.2. Fluorescence Titration

Fluorescence titration was performed in the range 400-700 nm, by adding heavy and toxic metals solutions (Hg2+, Cu2+, Ni2+, Cr3+, Pb2+, Ag+) to C-PC and recording the spectrum after each addition. The solution was excited at 488 nm by an argon laser of gas type and the fluorescence intensity was monitored at 635 nm. Metal free form of C-PC has a fluorescence maximum at 635 nm. The increase of the metal concentration causes the decrease in the peak amplitude. The fluorescence titration data on C-PC complexes with metal ions (Hg2+, Cu2+, Ni2+, Cr3+, Pb2+, Ag+) were plotted as binding isotherms and then analyzed by the Scatchard and Hill graphical methods[11-13]. The binding constants estimated from these isotherms are shown in Table 2. As it follows from Table 2 binding of Cr3+, Ni2+ and Cu2+ ions with C-PC is characterized by low affinity constant as compared with Hg2+, Ag+, Pb2+.

Table 2. *Energetics of metal ions binding with C-PC using fluorescence titration*

		Metal ion	Hg2+	Ag+	Pb2+	Cr3+	Cu2+	Ni2+
Fluorescence titration	Scatchard analysis	Constant of binding K×105M-1	11.4	5.2	2.8	1.0	0.6	0.44
		Gibbs free energy - ΔG°, kcal/mol	8.2	7.8	7.4	6.8	6.5	6.3
		χ^2	0.001	0.001	0.001	0.003	0.006	0.005
	Hill analysis	Constant of binding K×105M-1	12.0	5.5	2.3	0.90	0.74	0.48
		Gibbs free energy -ΔG°, kcal/mol	8.3	7.8	7.3	6.7	6.6	6.4
		R	0.98	0.94	0.97	0.96	0.95	0.96

The results indicate that value of calculated ΔG° of Cu2+, Ni2+ Cr3+, and Pb2+ ions is characteristic for hydrogen bonds (4.8÷7.4 kcal/mol) whereas Ag+, is almost on the verge of (7.2÷7.8 kcal/mol) and Hg2+ exceeds the energy of hydrogen bonding (8.2÷8.3 kcal/mol) (Table 2). In [5] for comparison was study the effect of selected heavy metal ions (Ag, Cr) on the photochemical activities and spectral properties in the photochemical activities in Spirulina platensis. Results indicate that among the selected metal ions, silver is a potent inhibitor of energy transfer and electron transport in this cyanobacterium. Heavy metals also suppressed the intensity of fluorescence emitted from phycocyanin at room temperature and induced blue shifts in the emission peak suggestive of changes in energy transfer within the phycobilisomes.

As it can be seen from the fluorescence data, the values of the binding constant K, determined both by the Scatchard and the Hill graphical analysis, are in good agreement, which naturally is valid for ΔG°. As to the fluorescence and absorption titration data, the values of K are in good agreement for Hg and Ag, but differ significantly for Cu, Ni and Cr, though the efficiency of interaction for different metals is arranged in the same sequence for the absorption titration as it is for fluorescence.

Absorption titration is generally impeded because of relatively small changes in absorption upon binding of metal complexes to proteins. These changes of absorption are especially small for ligands which have less affinity for C-PC (in our case Cu, Ni and Cr ions). Fluorescence titration provides a powerful technique for determination of constants of binding. The advantage of fluorescence over absorption measurements is in higher sensitivity, since fluorescence is detected against dark background, and selectivity of the signal. Moreover, to attain similar sensitivity, less amount of fluorescent ligand is required in fluorometry than in spectrophotometry. Fluorometry generally requires less disturbance of the system and allows of using less soluble ligands (in our case Hg, Pb, Cr). Fluorometry can also be used for samples with high turbidity. In addition, geometrical factors for fluorescence detection are less stringent than for absorption.

The above-mentioned may explain the difference in the constants of binding that are observed for absorption and fluorescent titration. However, it should be noted that during fluorescence titration the number of free metal ions is determined by an indirect method [8]. Therefore the equilibrium dialysis –a classical technique for determination of constants of binding was used in our further studies as an important criterion for estimation of the characteristics of binding of metal ions. To date, very few methods allow direct determination of protein-metal interactions.

3.3. Equilibrium Dialysis

In this section the energetics of Ag+, Pb2+, Cd2+, Ni2+, Cr3+, Cu2+ ions interaction with C-PC is defined via determination of the binding constants by equilibrium dialysis. The flame atomic-absorption spectral analysis was applied to register the equilibrium binding of metal ions to C-PC. The results are presented in Table 3. It is seen, the binding constants K determined by both the Scatchard and Hill methods are in good agreement, which is also valid for

ΔG0. The comparison of these results with data determined using optical methods indicate that the binding intensity for metal-C-PC complexes are arranged in the same sequence.

Table 3. *Energetics of metal ions binding with C-PC using equilibrium dialysis*

		Metal ion	Ag+	Cd2+	Pb2+	Cr3+	Cu2+	Ni2+
equilibrium dialysis	Scatchard analysis	Constant of binding K×105M-1	5.31	0.28	3.63	0.22	0.81	0.23
		Gibbs free energy - ΔG°, kcal/mol	7.78	6.05	7.56	5.9	6.68	6.18
		χ^2	0.008	0.001	0.001	0.007	0.005	0.004
	Hill analysis	Constant of binding K×105M-1	5.36	0.32	3.8	0.21	0.82	0.26
		Gibbs free energy -ΔG°, kcal/mol	7.8	6.13	7.59	5.88	6.68	6.3
		R	0.97	0.99	0.98	0.91	0.90	0.93

Obtained results using equilibrium dialysis are in good agreement with results using fluorescence spectroscopy in the case Ag+, but difference is observed for Cr3+, Ni2+ ions. The binding of salicylate ion to human serum albumin was discussed in 100mM potassium phosphate buffer using equilibrium dialysis and fluorescence titration methods [14]. This study serves to illustrate a likely complication in the study of protein-ligand interactions by these methods. In [15, 16] were studied influence of ionic strength on Cd2+_ and Pb2+_C-PC interactions. If take into account, that in the case equilibrium dialysis C-PC was dissolved in 20mM Na+ and in the case fluorescence titration C-PC was dissolved in water, hence, difference between thermodynamic parameters using equilibrium dialysis and fluorescence titrations methods may be explain inequality ionic strength. Proceeding from this, were studied effect of ionic strength on metal-C-PC complexes.

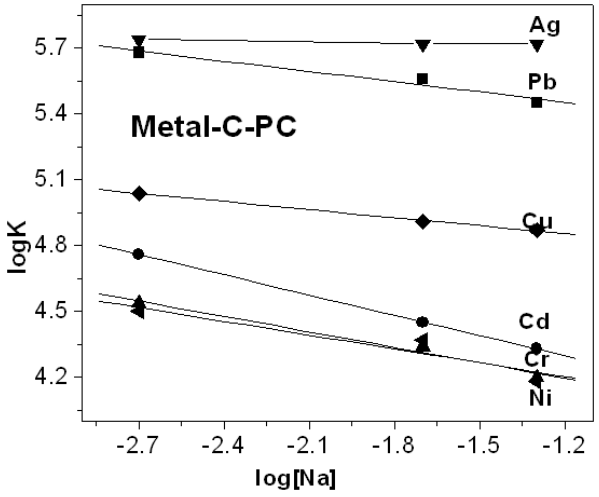

Figure 1. *Effect of ionic strength on the logK for metal-C-PC complexes*

In Figure 1 are presented influence of ionic strength on the binding constants using Scatchard analysis. The dependence of metal-C-PC complex stability on ionic strength of solution shows the competition of Na+ and metal ions for binding sites. However, the proximity of K values for Ag+, Pb2+ ions in the interval of Na+ concentrations under study indicates low competitive capacity of Na+ ions. Summarizing all obtained data, we can conclude that Ni2+, Cd2+,Cu2+, Cr3+, binding to C-PC has low affinity and C-PC is able to respond to the rise of Na+ level decreasing the affinity. Most effect of Na+ was obtained for Ni2+, and Cr3+.

4. Conclusion

Comparison of the binding constants of heavy metals with C-PC using difference optical and thermodynamic methods shown, that binding affinities of toxic and heavy metal ions for C-PC strongly depend upon the metal. The fluorescence and absorption titration data values of K are in good agreement for Hg and Ag, but differ significantly for Cu, Ni and Cr. Obtained results using equilibrium dialysis are in good agreement with results using fluorescence spectroscopy in the case Ag+, but difference is observed for Ni2+ and Cr3+ ions. Such effect in this case may be explain effect of ionic strength on the metal-C-PC binding constants.

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