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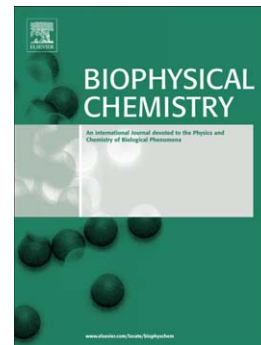
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**Kinetics of cyanide binding as a probe of local stability/flexibility of  
cytochrome *c***

by

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Abbreviations: cyt *c*, ferricytochrome *c*; MCD, magnetic circular dichroism

**Abstract**

Effect of anions of Hofmeister series (thiocyanate, perchlorate, iodide, bromide, nitrate, chloride, sulfate, and phosphate) on local and global stability and flexibility of horse heart ferricytochrome *c* (cyt *c*) has been studied. Global stability of cyt *c* was determined by iso/thermal denaturations monitored by change in ellipticity in the far-UV region and its local stability was determined from absorbance changes in the Soret region. Particularly, relative stability/flexibility of the Met80-heme iron bond has been assessed by analysis of binding of cyanide into the heme iron. Both global and local stabilities of cyt *c* exhibited monotonous increase induced by a change of anion from chaotropic to kosmotropic species. However, this monotonous dependence was not observed for the rate constants of cyanide association with cyt *c*. As expected more chaotropic ions induced lower stability of protein and faster binding of cyanide but this correlation was reversed for kosmotropic anions. We propose that the unusual bell-shaped dependence of rate constant of cyanide association is a result of modulation of Met80-heme iron bond strength and/or flexibility of heme region by Hofmeister anions independently on global stability of cyt *c*. Further, our results demonstrate sensitivity of cyanide binding to local change in stability/flexibility in heme region of cyt *c*.

**Keywords:** flexibility; protein dynamics; ligand binding; Hofmeister series

## Introduction

It is generally accepted that stability and conformational dynamics/flexibility are inversely related to each other [1,2]. Although the terms dynamics and flexibility in complex systems are usually interchangeable, they, in general, describe different properties. The dynamics describes motion(s) with a certain time scale, while the flexibility has no particular time reference but implies multiple structures that are of comparable energy and can be interconverted with a series of small changes [3]. Apparently, their interchangeable use follows from the fact that decreasing a barrier between two states increases rate of interconversion (dynamics) as well as accessibility of these two states (flexibility).

Stability and dynamics/flexibility of protein structure are intimately coupled to the properties (namely dynamics) of the solvent [4-8]. Structural fluctuations that occur on the fastest (~picoseconds) timescales permit the protein to sample a rugged energy landscape and ultimately facilitate slower, larger scale protein rearrangements [9]. Water interactions cause a softening of many of the free energy barriers between conformational sub/states [6,10]. The closest hydration shell surrounding proteins responsible for slaving of protein dynamics/flexibility to solvent motions can be efficiently affected by Hofmeister effect of ions [11-15]. The effect of anions on interfacial water structure next to proteins apparently may change the free energy barriers between the conformational sub/states [16].

While it is generally accepted that effect of ions on stability of proteins depends on the position of the ion in Hofmeister series [14,17-19], an effect of anions on dynamical properties of protein structure is not well understood. In fact, we have only recently showed that ions (both anions and cations) of Hofmeister series significantly affect dynamics/flexibility of the active site of NADH oxidase from *Thermus*

*thermophilus* resulting in unusual bell-shaped dependence of the enzyme activity on position of ions in Hofmeister series [20,21]. Moreover, Hofmeister effect of anions can affect not only transitions among substates (changing thus local dynamics/flexibility of the protein structure) but also “macroscopic” conformational transitions [22,23].

Recently, it was shown that binding of phosphate anions to cyt *c* destabilizes/increases flexibility of its heme region, specifically the Met80-heme iron bond [24]. This is surprising finding as there are well-known general stabilization properties of phosphate anions on protein structures and particularly on heme region of cyt *c* [25,26].

In the effort to investigate this finding in more details, we have systematically explored the Hofmeister effect of anions (0.5-2.0 M range) on local and global stability of cyt *c* examined by iso/thermal denaturation experiments and specifically on the Met80-heme iron bond examined by kinetics of cyanide binding to cyt *c*. Our findings show that cyanide binding to cyt *c* is useful probe for monitoring local stability/flexibility of heme region of cyt *c*. In the present work, we use the term flexibility in relation of cyanide binding to the protein as our results do not address fluctuations of polypeptide structure in specific timeframe.

## **Materials and Methods**

### *Materials*

Horse heart cytochrome *c*, type VI, was obtained from Sigma and used without purification. All other chemicals were purchased from Fluka and Sigma. Stock solutions of the salts were filtered before use.

### *Absorption measurements*

Absorption experiments were made with a Varian Cary Bio 100 spectrophotometer equipped with a Peltier element. Thermally induced unfolding of cyt *c* was monitored at 396 nm. Data were fitted by using the equation:

$$A_{obs} = \frac{A_N + m_N T + (A_U + m_U T) \exp\left[\frac{\Delta H_{trs}}{R} \left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right]}{1 + \exp\left[\frac{\Delta H_{trs}}{R} \left(\frac{1}{T} - \frac{1}{T_{trs}}\right)\right]} \quad (1)$$

where  $A_{obs}$  is observed absorbance at temperature  $T$ ,  $A_N$  and  $A_U$ , and  $m_N$  and  $m_U$ , represent intercepts and slopes of the native (predenaturation) and unfolded (postdenaturation) baselines, respectively, and  $\Delta H_{trs}$  is the enthalpy at the transition temperature  $T_{trs}$ .

#### *Circular dichroism measurements*

The isothermal denaturation of cyt *c* was followed by measurement of the ellipticity using a Jasco J-810 spectropolarimeter. The circular dichroism (CD) measurements were done in a solution of 7-9  $\mu\text{M}$  cyt *c*, 5 mM sodium cacodylate (pH 6.0), 1  $\mu\text{M}$  potassium ferricyanide, and the salt. For measurements in the 250-450 nm region a 1 cm pathlength cuvette was used, and for the 190-250 nm region a 0.1 cm pathlength cuvette was used. The urea-induced spectral changes were detected at 222 nm (no salt,  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_2\text{SO}_4$ , KCl), 225 nm ( $\text{NaClO}_4$ ), 235 nm (KBr) and 417 nm ( $\text{NaNO}_3$ , NaI, KSCN). The different wavelengths were used to minimize the contribution from the salt in the CD signal. Measurements were performed at 24 °C.

The Gibbs free energy of unfolding,  $\Delta G^\circ$ , that expresses the protein stability and the  $m$  value, relating to the amount of protein surface exposed to solvent upon unfolding [27], were fitted using following equation:

$$\theta = \frac{\Delta\theta_N + m_N[D] + (\Delta\theta_U + m_U[D]) \exp\left(\frac{-\Delta G^\circ + m[D]}{RT}\right)}{1 + \exp\left(\frac{-\Delta G^\circ + m[D]}{RT}\right)} \quad (2)$$

where  $\theta$  is observed ellipticity at the denaturant concentration  $[D]$ ,  $\Delta\theta_N$  and  $\Delta\theta_U$  represent the intercepts of the pre- and postdenaturation baselines, respectively, and  $m_N$  and  $m_U$  represent the slopes of the pre- and postdenaturation baselines, respectively. This equation takes into account the linear dependence of the pre- and postdenaturation region of the unfolding curve [28].

The pH values were measured directly in the cuvettes using a Sensorex glass microelectrode, before and after measurements.

#### *Near-infrared magnetic circular dichroism measurements*

Data were collected in JASCO J-730W spectropolarimeter at room temperature. All samples of 1 mM oxidized cytochrome *c* were in D<sub>2</sub>O buffers. Buffers were prepared by freeze and dry water solutions and solubilization of solid residues with the adequate amount of D<sub>2</sub>O.

#### *Kinetic studies*

The kinetic measurements were performed with a Specord S 300 UV-VIS diode-array spectrophotometer equipped with a Peltier element. The protein was fully oxidized prior to the kinetic measurements by the addition of 1  $\mu$ l of 10 mM potassium ferricyanide per 1.5 ml of sample volume. The final protein concentration was  $\sim 9 \mu$ M. The samples were in 50 mM sodium cacodylate buffer, pH 6.0, in the presence or absence of 0.5, 1.0, 1.5 and 2.0 M salts (NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, NaCl, NaBr, NaNO<sub>3</sub>, NaI, NaClO<sub>4</sub> and KSCN) at  $24.0 \pm 0.1$  °C.

A freshly prepared 0.5 M sodium cyanide stock solution was adjusted to pH 6 with concentrated HCl and used within four hours. In a typical experiment the



reaction was initiated by quickly mixing a given volume of 0.5 M HCN with the solution of cyt *c*. The final concentration of HCN in the cuvette ranged from 10 to 90 mM. The formation of the complex was followed at 418 nm, which is the maximum in the difference spectrum between the cyt *c*-CN<sup>-</sup> complex and the free (unliganded) cyt *c*. The absorbance data as a function of time were fitted to a single exponential function of the form:

$$A_t = A_0 + A_1 \exp(-k_{\text{obs}}t) \quad (3)$$

where  $A_t$  is the absorbance at time  $t$ ,  $A_0$  is the final absorbance at time  $t = \infty$  and  $A_1$  is the total change in absorbance between  $t = 0$  and  $t = \infty$ , and  $k_{\text{obs}}$  is the observed pseudo first-order rate constant. The association rate constant,  $k_a$ , was estimated from the slope of the best-fit plot of the pseudo first-order rate constants versus the concentration of HCN according to equation [29]:

$$k_{\text{obs}} = k_d + k_a[\text{HCN}] \quad (4)$$

where  $k_d$  represents the dissociation rate constant of the cyt *c*-CN<sup>-</sup> complex.

The pH of the solution was determined after each kinetic experiment.

## Results

To study the effect of Hofmeister anions on stability of cyt *c*, and to avoid complications due to specific anion binding to cyt *c*, we used high ( $\geq 0.5\text{M}$ ) concentrations of anions when Hofmeister effect prevails [14,30,31]. The native conformation of cyt *c* is unaffected in high ionic strength [32]. This is evidenced by an absence of significant spectral change in the Soret region that monitors close environment of sensitive probe – heme, as well as the band 695 nm which monitors integrity of charge-transfer bond between sulfur of Met80 and heme iron (Fig. 1).

The stability of cyt *c*, expressed in Gibbs free energy ( $\Delta G^\circ$ ), in the presence of

different salts and their concentrations (0.5 and 1.0 M) was determined by isothermal urea denaturation monitored by ellipticity changes in the far-UV or the Soret region of CD spectra (Table S1). A dependence of  $\Delta G^\circ$  values on the position of the anions in Hofmeister series shows a monotone increasing trend from chaotropic to kosmotropic anions (Fig. 2A) in accordance with generally accepted effect of Hofmeister anions on protein stability [18].

To investigate a local stability of cyt *c* on Hofmeister anions, we determined thermal stability of the heme region monitored by absorbance of cyt *c* in the Soret region. The dependence of transition temperatures on the position of the anions in Hofmeister series is shown in Figure 2B. The thermal denaturation of cyt *c* was also determined by monitoring an ellipticity change in the far-UV region of CD spectra. This provided us with information about stability of the secondary structure of protein reflecting the global stability of cyt *c* (Fig. 2B). Both local (absorbance in the Soret region) and global (ellipticity in the far-UV region) stabilities of cyt *c*, expressed by transition temperatures, increase in a monotone way from chaotropic to kosmotropic anions. Resemblance of thermal transition temperatures measured by absorbance and ellipticity points to two-state character of the thermal transition of cyt *c* at given conditions.

To examine our hypothesis that modulated stability of heme region, particularly the Met80-heme iron bond, is reflected in kinetics of cyanide binding to cyt *c*, we studied effect of Hofmeister anions on association rate of interaction between cyanide and the protein. In fact, the association rate constant of cyanide binding with cyt *c* strongly depends both on a position of anion in Hofmeister series (Fig. 3) and anion concentration (Fig. 3B, Table S3). The progress of the reaction of cyanide binding into the protein was fitted with monophasic exponentials at all

studied conditions (Fig. 3A). Observed pseudo first-order rate constants,  $k_{\text{obs}}$ , obtained from these fits, were plotted vs concentration of added HCN. From the linear dependences (inset Fig. 3A) association,  $k_{\text{a}}$ , and dissociation,  $k_{\text{d}}$ , rate constants of cyanide binding were determined in accordance with the equation (4). Because the precision of  $k_{\text{d}}$  values is negatively affected by an extrapolation to zero concentration of the ligand (Table S3), analysis of the  $k_{\text{d}}$  values was omitted in the further work. The plot of  $k_{\text{a}}$  vs type of anion (Fig. 3B) shows the strong dependence of this parameter on the position of the anion in Hofmeister series: while neutral (from Hofmeister effect standpoint) anions has only minimal effect on the  $k_{\text{a}}$  values, chaotropic and in less extent also kosmotropic anions led to an increase in the association rate constant of cyanide binding with cyt *c*. The effect of chaotropic anions on the association rate constant is even more pronounced at 2 M concentration when the rate constant in the case of KSCN increases  $\sim 40$  times in comparison with that in the absence of salt (Table S3). The effect of kosmotropic anions on the association rate was not possible to analyze at such high salt concentrations due to their limited solubility at given conditions. It seems that penetration of cyanide through the protein matrix and/or cyanide binding to heme iron are alleviated in the presence of chaotropic and kosmotropic anions in comparison with the neutral anions. This is demonstrated as a minimum of  $k_{\text{a}}$  value in the presence of chloride anions in the plot shown in Figure 3B.

Unexpected enhancement of the observed rate of cyanide binding in both kosmotropic (phosphate) and chaotropic (thiocyanate) solutions relative to the plain buffer and neutral Hofmeister salt (sodium chloride) prompted us to investigate the stability of axial coordination of cyt *c* under these conditions. Near-infrared magnetic circular dichroism (MCD) spectroscopy is one of the tools for an identification of the

axial ligands in the low spin heme proteins [33,34]. As Figure 4 illustrates MCD spectra of cyt *c* in the buffer and in the presence of 1M chloride are identical. On the other hand, the presence of either 1 M phosphate or 1M thiocyanate results in the decrease of the band intensity at ~1730 nm. This decrease indicates that the native axial coordination of His-Fe-Met in cyt *c* is partially disrupted by both anions.

## Discussion

### *Factors affecting cyanide binding to hemoproteins*

The major determinants that govern the affinity of the cyanide for hemoproteins such as metmyoglobins and methemoglobins that have heme iron connected with bulk solvent through channel are: (i) the ease of displacement of the sixth ligands (in the cases of these proteins: water molecule), (ii) the acid dissociation constant of HCN inside the protein, and (iii) steric hindrance and electrostatic interactions at the sixth coordination position [35,36]. The observed association rate constant  $k_a$  is proportional to the major parameters that can be expressed by the simplified equation:  $k_a \sim k_{\text{diff}}Q_{\text{CN}}Q_{5c}$ , where  $k_{\text{diff}}$  is the rate of diffusion of HCN through the protein matrix,  $Q_{\text{CN}}$  is the equilibrium distribution of the protein molecules that have a cyanide anion in the heme pocket poised to bind to the heme iron once the sixth ligand dissociates, and  $Q_{5c}$  is the equilibrium distribution of penta-coordinate heme.

In the hemoproteins such as cyt *c*, where there is no apparent channel connecting bulk solvent with heme iron, there might be important one more determinant: a barrier caused by protein environment surrounding the heme. However, Lakowicz and Weber showed that small molecules such as oxygen and even soft iodide anion diffusion rate through proteins,  $k_{\text{diff}}$ , is very fast, diminished only by two

orders of magnitude in comparison with diffusion rate in water [37]. On the other hand, example supporting an existence of the barrier induced by protein environment is ferricytochrome *c'* from *Chromatium vinosum*. In this case, kinetics of cyanide binding to the heme iron is despite the fact that it contains penta-coordinated heme, i.e.  $Q_{5c} \sim 1$ ,  $10^2$  slower than to horse heart cyt *c* (hexa-coordinated heme) and more than  $10^5$  slower than that to metmyoglobin and methemoglobin [38]. Assuming similar value of  $pK_a$  constant of HCN in heme region on cyanide binding in the proteins, the association rate has to be affected mainly by  $k_{diff}$ . Taking into account results of Lakowicz and Weber, the value of  $k_{diff}$  is proportional to accessibility of heme iron. This is arguably modulated by flexibility of the crowded heme region of ferricytochrome *c'* from *Ch. vinosum* or stability/flexibility of heme region, particularly of bond between heme iron and the sixth ligand in the case of horse heart cyt *c*.

#### *Effect of (Hofmeister) salts on properties of cyt c*

Advantage of the modification of solvent properties by Hofmeister salts is that solvent modification affects only stability/flexibility of the sixth ligand without an effect on the other determinants of the affinity of cyanide for heme. The unaffected spectral properties of cyt *c* even at high concentrations of Hofmeister anions imply an absence of any major conformational change (Fig. 1). Structural studies performed by NMR [39,40], X-ray crystallography [32], and ultraviolet resonance Raman spectroscopy [41] also indicate only minor and locally distributed changes on Met80 side of the heme in the structure of cyt *c* due to high ionic strength. On the other hand, small-angle X-ray scattering measurements indicate decrease of the radius of gyration of cyt *c* by  $\sim 8\%$  due to neutralization of high positive charge of cyt *c* surface by high ionic strength of solvent [42]. One might expect that the neutralization of the

repulsive positive charges in the protein structure will stabilize the structure either locally and/or globally. However, the effect of high ionic strength on cyt *c* at neutral pH region strongly depends on the nature of ions in accordance with the Hofmeister effect. While it is generally accepted that chaotropic, soft anions efficiently screen charge interactions in proteins due to their tendency to bind to hydrophobic patches on protein surface [43,44] and to induce a salt in effect on the peptide group [19], the situation is more complex for kosmotropic anions. Kosmotropic anions are strongly hydrated and therefore, considered to be excluded from protein surface [45-47]. Moreover, according to Collins' "law of matching water affinities" kosmotropic anions should have low affinities for chaotropic ammonium groups on protein surface [48,49]. This hypothesis was supported experimentally by investigation of ion effects on interfacial water structure next to macromolecules with vibrational sum frequency spectroscopy [50]. In accordance, it was shown that Hofmeister effect of anions on stability of proteins is nearly independent on charge of protein surface implicating an absence of specific electrostatic interactions [43]. On the other hand, it was experimentally shown the stabilization effect of kosmotropic anions through their specific interactions with protein surface [30,51,52], and recent molecular dynamic simulations show tendency of sulfate anions to be strongly attached to the proteins and generally, polycationic surfaces [53-55]. Cyt *c* contains high affinity binding site for sulfate and phosphate anions localized at the heme crevice [25]. Therefore, it is likely that the effect of kosmotropic anions on cyt *c* might be combined effect of specific (high affinity site) and nonspecific (Hofmeister effect) interactions.

*Chaotropic and kosmotropic anions modulate stability/flexibility of heme region of cyt c.*

In accordance with our findings in this work, a plausible explanation for an

unusual bell-shaped dependence of  $k_a$  vs anions of Hofmeister series, particularly the increase of  $k_a$  values in the presence of kosmotropic anions provides MCD data. Data show that axial coordination of His-Fe-Met is partially disrupted not only by chaotropic thiocyanate but also by phosphate anions. Thus it appears that despite kosmotropic effect of phosphate anions on global stability of protein at the same time these ions impose a local destabilization of the iron axial coordination either through nonspecific Hofmeister effect or through specific interaction with high affinity site at the heme crevice [25,26]. To reconcile observed increased global and local stabilities with MCD spectrum in the presence of phosphate anions, we suggest that the decreased MCD band is an expression of an increased flexibility in the heme region. The MCD spectrum is then average spectrum of several substates of heme region of cyt *c* in solutions containing phosphate anions.

In fact, our MCD data are consistent with the results of the work of Shah and Sweitzer-Stenner [24]. The authors studied structural changes of horse heart cyt *c* induced by ionic strength and anion binding. They concluded that binding of phosphate anion (kosmotrope) to cyt *c* stabilizes the protein, however, at the same time, increases heterogeneity of the 695-nm band that indicates a higher flexibility of the protein segment that contains Met80 as a result of entropic stabilization [56]. In fact, it was shown that charge-transfer band at 695 nm in ferricytochrome *c* spectrum is composed of at least three bands, assignable to different substates [57] with different thermal stabilities [58]. It should be pointed out that iso/thermal denaturation experiments showed that both local and global stabilities of cyt *c* increased in the presence of kosmotropic anions. On the other hand, only cyanide binding “feels” increased flexibility of Met80-heme iron linkage in the presence of kosmotropic anions in accordance with recent findings of Shah and Sweitzer-Stenner

[24]. The increased flexibility is therefore paradoxical consequence of a strengthening of Met80-heme iron bond that is in all substates likely accompanied by changed equilibrium distribution between penta- and hexa-coordinated hemes in favor of the former state as is indicated by MCD spectra. On the other hand, the effect of chaotropic anions on the heme region of cyt *c* can be looked at as solvent-protein interaction induced dynamic rearrangement which weakens the strength of methionine sixth axial ligation and permits its replacement by exogenous strong field ligands.

*Physiological implications of limited flexibility of cyt c.*

Apparently, anions can modulate stability/flexibility of heme region of cyt *c* with a consequence of different accessibility of heme iron from the bulk solvent with likely of functional significance [24]. The minimum value of  $k_a$ , i.e. the smallest accessibility of heme region to solvent, at low (physiological) ionic strength *in vitro* might therefore have physiological role because an exposure of heme as well as modified mobility in heme region affect both redox properties [59,60] and stability [61] of cyt *c*. In fact, the dynamics of dissociation/association of Met80-heme iron bond ( $\sim 30 \text{ s}^{-1}$ ) appears to be conserved throughout evolution in class I of c-type cytochromes [62]. Interestingly, it was shown in bacterial cyt *c* that dynamics of the hinge region (in horse cyt *c* includes residues 70-85), responsible for dissociation/association of Met80-heme iron, facilitate its dissociation from its physiological partner suggesting thus a role of this movement in turnover under physiological conditions [63].

*Conclusions.*

In conclusion, the unusual dependence of cyanide association rate constant on the position of anions in Hofmeister series demonstrates sensitivity of cyanide



binding on modulated stability of Met80-heme iron either as a result of global destabilization of cyt *c* by chaotropic anions or an unusual entropic stabilization by kosmotropic anions that both lead to higher local flexibility of its heme environment.

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ACCEPTED MANUSCRIPT

**Figure legends:**

**Figure 1.** Absorption spectra of cyt *c* in the presence of 1.0 M salts (NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>SO<sub>4</sub>, NaCl, NaClO<sub>4</sub>) at pH 6.0 (50 mM sodium cacodylate buffer). Dashed line represents spectrum in the absence of salt. Protein concentration was 14 μM (350-580 nm) and 400 μM (620-800 nm).

**Figure 2.** A: Dependence of stability of cyt *c* expressed in free Gibbs energy on the position of anions in Hofmeister series at 0.5 M (white boxes), 1.0 M (grey boxes), and in the absence of salt (black box). B: Dependence of stability of cyt *c* expressed as a midpoint of the thermal transitions obtained from absorbance changes in the Soret region (white boxes) and ellipticity changes in the far-UV region (grey boxes) in the presence of 1.0 M salts and in the absence of salt. Standard errors in determination of  $T_{\text{trs}}$  were  $\pm 0.5$  °C or less in all cases.

**Figure 3.** A: Time dependence of the absorbance change monitored at 418 nm during the reaction of cyt *c* with 0.09 M HCN in the presence of 1.0 M KSCN (white circles) and 1.0 M NaH<sub>2</sub>PO<sub>4</sub> (black circles) in 50 mM sodium cacodylate, pH 6.0, at 24 °C. The data obtained were fitted to a single exponential function. Inset: Dependence of the observed rate constant on HCN concentration for cyt *c*-CN<sup>-</sup> complex formation in the presence of 1.0 M KSCN (white squares) and 1.0 M NaH<sub>2</sub>PO<sub>4</sub> (black squares). B: Dependence of association rate constants of cyanide binding into cyt *c* on the position of anions in Hofmeister series at 0.5 M (white boxes), 1.0 M (grey boxes), and in the absence of salt (black box).

**Figure 4.** Influence of salts on MCD spectrum of cyt *c*: in the presence of 1 M sodium chloride (solid line), 1 M sodium phosphate (dashed line) and 1 M sodium thiocyanate (dotted line). The MCD spectrum of cyt *c* in the buffer is identical with that in the presence of 1M sodium chloride (solid line). The buffer in each case was 50 mM cacodylate, pH 6.0.

Figure 1

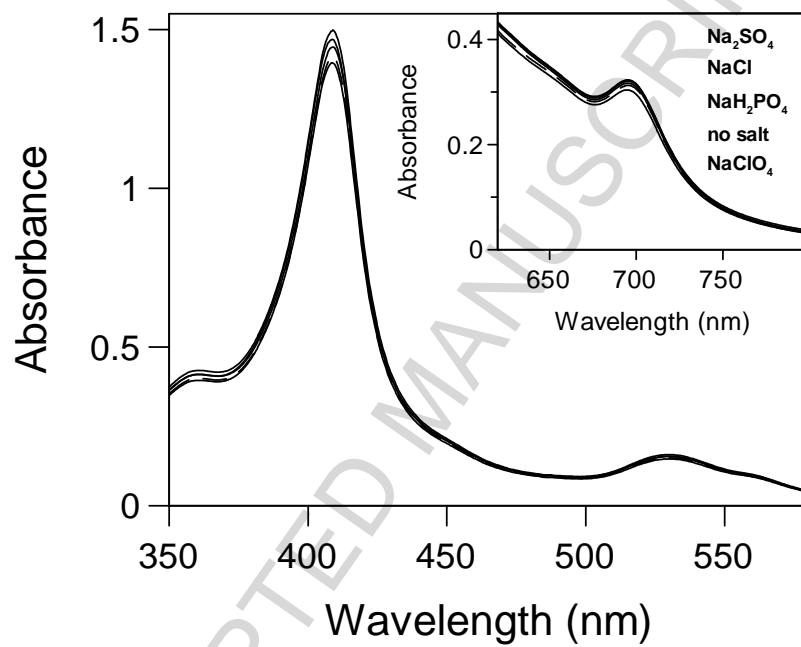


Figure 2

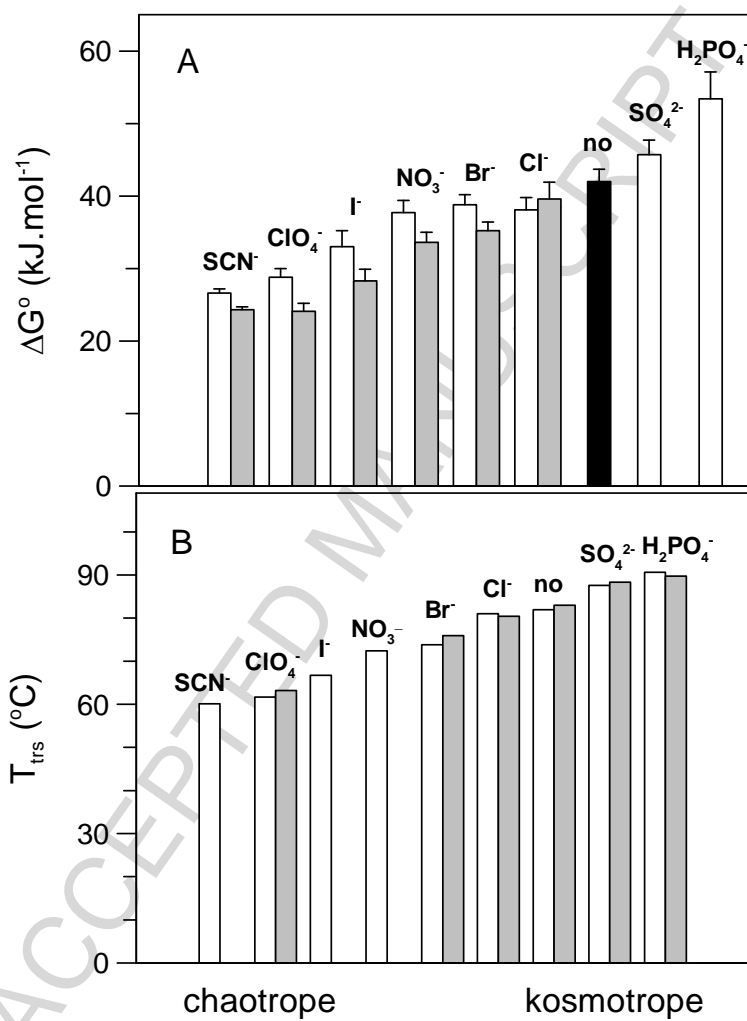


Figure 3

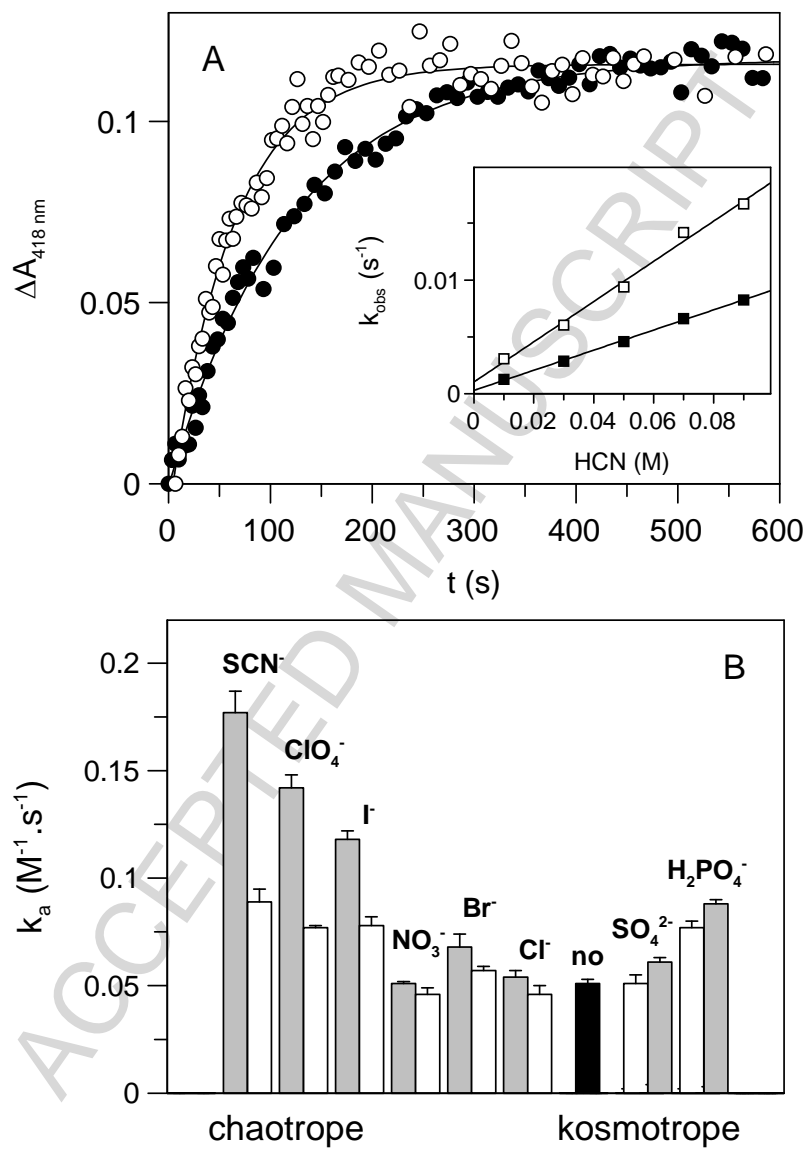


Figure 4

