Effect of Nucleotides on Thermal Stability of Ferricytochrome C

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Abstract. The effect of nucleotides on the structure and thermal stability of ferricytochrome c was studied by differential scanning calorimetry. The association of cytochrome c with ATP and ADP resulted in a decrease in the denaturation temperature of cytochrome c by 7°C and 4°C, respectively, at pH 7.0. AMP did not change the denaturation temperature of cytochrome c at pH 7.0. The ratio between van't Hoff and calorimetric enthalpy of denaturation accounts for the fact that cooperative denaturation of 3–4 molecules of cytochrome c occurred in the presence of ATP at the pH range from 5 to 9. ADP gave rise to the interaction of 2–3 molecules of ferricytochrome c at pH 6–7.5, and AMP did not affect the interaction of protein molecules. Cytochrome c alone also associated at pH 7.5–10. At physiological ionic strength, pH 7.0, only ATP induced an association of ferricytochrome c molecules. No intermolecular interaction of ferricytochrome c molecules was observed at concentrations of NaCl higher than 0.2 mol/l not even in the presence of ATP.

Key words: Cytochrome c — Nucleotides — Microcalorimetry

Introduction

Cytochrome c in eukaryotes is a component of the mitochondrial chain which acts in the electron transport from cytochrome c reductase to cytochrome c oxidase (the membrane embedded complexes III and IV). The proton gradient created during the electron transfer in the respiration chain is used in ATP synthesis in the process of oxidative phosphorylation (Capaldi 1982). Cytochrome c is a highly basic protein able to interact with anions (negatively charged molecules) (Aviram 1973). The interaction between nucleotides and cytochrome c is an issue of great interest. Some efforts in this respect have been spent by Margoliash et al. (1970) who studied the association of ATP and ADP with cytochrome c with respect to the electrophoretic mobility of this protein. Kayushin and Ajipa (1973) by means of NMR observed the

association of cytochrome c with ATP or ADP, but not with AMP. The nucleotides have been shown to affect the kinetics of the electron transfer from cytochrome c to cytochrome c oxidase (Bisson et al. 1987). The inhibitory power decreases from ATP to ADP to AMP. Corthesy and Wallace (1986) found that 2.47 ATP molecules bound to one molecule of ferricytochrome c. They observed a higher affinity of ATP to cytochrome c to an area near to Arg 91. The specificity of binding to this place is a function of nucleotide phosphorylation rather than a characteristic of the nucleoside. Craig and Wallace (1991) showed that the presence of triphosphate group primarily determines high affinity binding, but some effect is contributed by the nucleoside moiety as well. Triphosphates interact with cytochrome c at the same region as Arg 91, and this binding facilitates the protein aggregation (Whitford et al. 1991). The degree of aggregation change from tetramers to dimers can be regulated by ionic strength. Goto et al. (1991) showed that ATP induces a conformation change in cytochrome c at pH 2, and a compact structure with a significant amount of α-helix is formed. In our previous observations (Antalík et al. 1992a,b) it was found that cytochrome c created complexes with polyanions such as heparin, polyglutamic acid, polynucleotides in which some properties of the protein were changed. Bágeľová et al. (1994) could show that during thermal denaturation two or three molecules of cytochrome c are associated in the presence of heparin.

In this paper the results of a study are presented of ATP, GTP, UTP, CTP, ADP and AMP effects on the thermal stability of cytochrome c at a wide pH range, as investigated by differential scanning calorimetry. A strong aggregation of ferricytochrome c with ATP and other triphosphatenucleotides in low ionic strength media was found.

Abbreviations used: AMP, adenosine-5′-phosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; GTP, guanosine-5′-triphosphate; UTP, uridine-5′-triphosphate; CTP, cytidine-5′-triphosphate; DSC, differential scanning calorimetry; ΔH_{cal}, calorimetric enthalpy; ΔH_{vH}, van't Hoff enthalpy; T_m, temperature of transition expressed in Celsius; $T_{m,\mathrm{K}}$, temperature of transition expressed in Kelvin; c.c., correlation coefficient.

Materials and Methods

Horse heart cytochrome c (type III, Sigma Chemical Co.) was used without any further purification. Adenosine-5′-phosphate, adenosine-5′-diphosphate·Na_2-salt, adenosine-5′-triphosphate·Na_2-salt, cytidine-5′-triphosphate·Na_2-salt, uridine-5′-triphosphate and guanosine-5′-triphosphate·Na_2-salt were purchased from Serva. Before using, cytochrome c (100–110 μmol/l) was converted into its fully oxidized form by adding $K_3Fe(CN)_6$ (50 μmol/l). The measurements were performed in 2 mmol/l buffers – glycine/HCl, acetate, HEPES, phosphate and glycine/NaOH, respectively, at the pH ranging from 2 to 10.5.

The concentration of cytochrome c was determined spectrophotometrically, using the absorbance coefficient of $\epsilon_{550}^{red-ox} = 21$ (mmol/l)$^{-1}$.cm^{-1}. Gel chromatography was performed on Sephadex G-75 (Pharmacia) columns ($l = 20$ cm, ø= 1.3 cm). As markers lysozyme, trypsinogen and bovine albumin (from Sigma) were used. The protein sample (1 mg) was added in a volume of 0.2 ml. The flow rate was 1.2 ml/min.

Calorimetric measurements were carried out by means of a scanning microcalorimeter DASM-4 connected to a personal computer. The scan rate was 1 deg/min for all experiments. All the calorimetric curves were corrected using an instrumental baseline obtained by heating the buffer.

The pH of the samples was determined before the measurements, and was checked again thereafter. Only measurements were taken into consideration with a pre- vs. post-heating pH difference less than 0.1.

The calorimetric enthalpy was derived from the area of the heat absorption peak, using electric calibration (Privalov and Khechinashvili 1974). The peak area was taken as the area limited from above by the heat absorption curve and from below by the heat capacity values of native and denatured protein obtained by linear extrapolation of heat capacity before and after the process to mid-transition at the transition temperature T_m.

The van't Hoff enthalpy was calculated simultaneously from the same calorimetric curve according to $\Delta H_{vH} = 4RT_{m,\mathrm{K}}^2 C_{p\,\max}/Q_t$, where $C_{p\,\max}$ is the height of the heat capacity peak at denaturation temperature, $T_{m,\mathrm{K}}$, and Q_t is the peak area in energy units. Strictly speaking, this equation can only be applied to a reversible process, but it has been shown that DSC thermograms for the thermal denaturation of several proteins can be interpreted in terms of the van't Hoff equation, in spite of calorimetric irreversibility (Edge et al. 1985; Manly et al. 1985; Hu and Sturtevant 1987).

The ratio $\Delta H_{vH}/\Delta H_{\mathrm{cal}}$ (r) equals 1 if the transition is of the two-state type. For a process with intermolecular cooperation $\Delta H_{vH}/\Delta H_{\mathrm{cal}} > 1$ and value r an estimation is provided of the number of molecules included in the cooperative units (Mateo 1984).

Results

Fig. 1. shows thermograms of ferricytochrome c in the presence of various concentrations of ATP at low ionic strength (2 mmol/l HEPES, pH 7.0). The addition of ATP resulted in a decrease of its denaturation temperature, T_m. A half-maximum change of transition temperature was 0.3 mmol/l ATP (curve 1, Fig. 1, inset). The saturated concentration of ATP was 2 mmol/l, and in these conditions the T_m of cytochrome c was 75 °C and the calorimetric enthalpy of transition $\Delta H_{\mathrm{cal}} = 280$ kJ/mol. In comparison with cytochrome c, ATP in these media decreased the transition temperature by 9.7 °C whereas calorimetric enthalpy slightly increased, by about 50 kJ/mol. Curve 2 in Fig. 1 shows that the saturating concentration of ADP (4 mmol/l) decreased the transition temperature to 78 °C, and a half-maximum temperature change was achieved by the addition of ADP in a concentration of 0.5 mmol/l. On the other hand (curve 3, Fig. 1 inset) AMP did not affect the transition temperature of cytochrome c at concentrations lower than 4 mmol/l.

The thermal transition of ferricytochrome c in the presence of nucleotides is complicated by irreversible postdenaturation changes which prevent the system

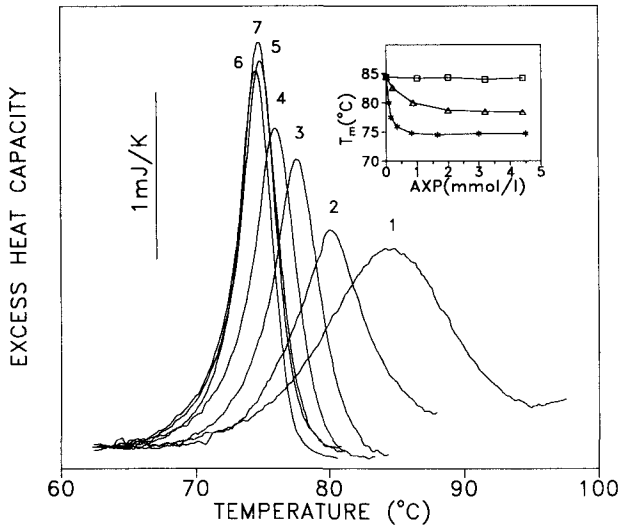


Figure 1. DSC scans of ferricytochrome c in 2 mmol/l HEPES, pH 7.0, at increasing concentrations of ATP; *1* – 0; *2* – 0.09; *3* – 0.16; *4* – 0.36; *5* – 0.82; *6* – 1.65; *7* – 4.5 mmol/l ATP. The protein concentration was 100 μmol/l. **Inset**: The dependence of denaturation temperature T_m on nucleotide concentration: (□) AMP, (△) ADP, (∗) ATP; 2 mmol/l HEPES, pH 7.0.

from returning to the initial state upon simple cooling. Since the observed heat effect, T_m and the shape of the melting curve did not depend significantly on the heating rate (0.5–2°C/min) it is supposed that there is local equilibrium in the system, and that denaturation of cytochrome c is not kinetically controlled (Privalov and Medveď 1982; Sanchez-Ruiz et al.1988). The shape of the melting curve at the saturating concentration of ATP did not depend on the concentration of cytochrome c (not shown), and the thermograms were symmetric. It can be supposed that the association or dissociation are not accompanied by the process of denaturation observed with DSC (Sturtevant 1987).

Cytochrome c is a simple globular protein (Takano and Dickerson 1981), and it was shown under conditions of low pH (2.2–3.6) that its thermal transition may be characterized as a two-state process (Privalov and Khechinashvili 1974). A high number of positive charges in this medium prevent intermolecular interactions of cytochrome c, and the ratio between van't Hoff and calorimetric enthalpy of transition, r (coefficient of cooperativity) is close to one. At low ionic strength, pH 7.0, a ratio of 1.5 was established. This ratio suggests that, under these conditions, partial interaction of cytochrome c molecules occurred. In comparison with ADP and AMP, the saturated concentration of ATP resulted in the greatest narrowing

of the denaturation peak. The cooperative coefficient r, increases with the number of phosphate groups bound to the ribose. While in cytochrome c or in the presence of AMP 1–2 molecules of cytochrome c interact, in the presence of ADP the interaction occurs between 2–3 molecules, and in the presence of ATP between 3–4 molecules of cytochrome c (Fig. 2 inset).

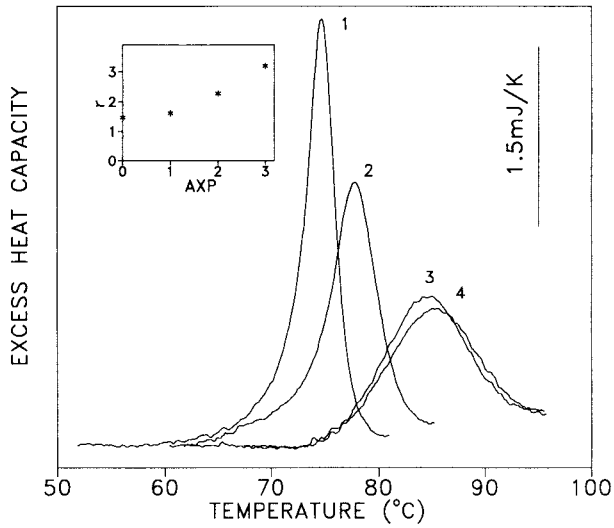


Figure 2. DSC scans of ferricytochrome c with nucleotides in 2 mmol/l HEPES, pH 7.0; *1* – ATP; *2* – ADP; *3* – AMP; *4* – cytochrome c alone. The protein concentration was 100 μmol/l, nucleotide concentration was 4.5 mmol/l. **Inset**: The dependence of cooperativity coefficient r ($\Delta H_{vH}/\Delta H_{\mathrm{cal}}$) on phosphate groups number in nucleotide; 2 mmol/l HEPES, pH 7.0.

Table 1 presents some thermodynamic parameters of ferricytochrome c in the presence of nucleotides. By the action of saturated concentrations of triphosphates, the thermal transition temperature is decreased at pH 7.0 to around 75 °C. The calorimetric enthalpy of thermal transition slightly increased to 260–300 kJ/mol when compared with the medium containing only phosphate (ΔH_{cal} = 241 kJ/mol). However, van't Hoff enthalpy was strongly developed. It is evident that ATP had the strongest effect on ΔH_{vH} (900 kJ/mol). A half-maximum temperature change was achieved by adding $\sim$ 0.3 mmol/l ($c_{1/2}$) of any of the triphosphates tested. ADP or AMP had significantly lower effectiveness. The decrease of T_m and the degree of intermolecular interaction depended on the number of phosphate groups in the nucleotide molecule. At pH 7.0, the phosphate groups are partially dissociated and the number of negative charges, which can interact with

Table 1. Thermodynamic parameters of ferricytochrome c in the presence of phosphate (Pi) and nucleotides (NXP) at pH 7.0.

NXP	T_m [°C]	ΔH_{cal} [kJ/mol]	ΔH_{vH} [kJ/mol]	$c_{1/2}$ [mmol/l]
Pi	85.6	241	380	–
ATP	75.0	280	900	0.3
GTP	74.6	260	663	0.2
CTP	75.9	300	632	0.3
UTP	76.7	275	631	0.3
ADP	78.5	280	580	0.5
AMP	84.3	290	500	–

Conditions: 2 mmol/l phosphate buffer, 5 mmol/l nucleotides, 110 μmol/l cytochrome c.

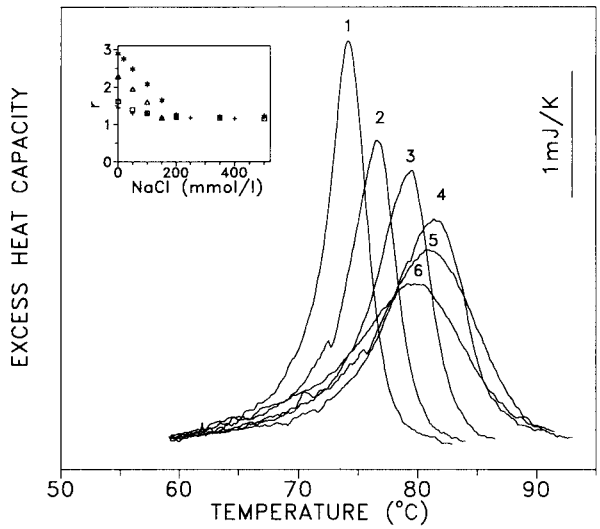


Figure 3. DSC scans of ferricytochrome c with ATP in 2 mmol/l HEPES, pH 7.0 at increasing concentrations of NaCl; *1* – 0; *2* – 0.02; *3* – 0.05; *4* – 0.1; *5* – 0.2 mol/l and *6* – 0.5 mol/l NaCl. The protein concentration was 100–105 μmol/l, that of ATP was 4 mmol/l. **Inset**: The dependence of cooperativity coefficient r on NaCl concentration (+) cytochrome c alone, (□) AMP, (△) ADP, (∗) ATP.

positive charges on the surface of cytochrome c increases from AMP to ATP. The electrostatic origin of interactions of cytochrome c with ATP is illustrated in Fig. 3. An increase in NaCl concentration induced a shift of T_m towards higher values, and simultaneously the sharpness decreased. As shown in Fig. 3, an increase of NaCl

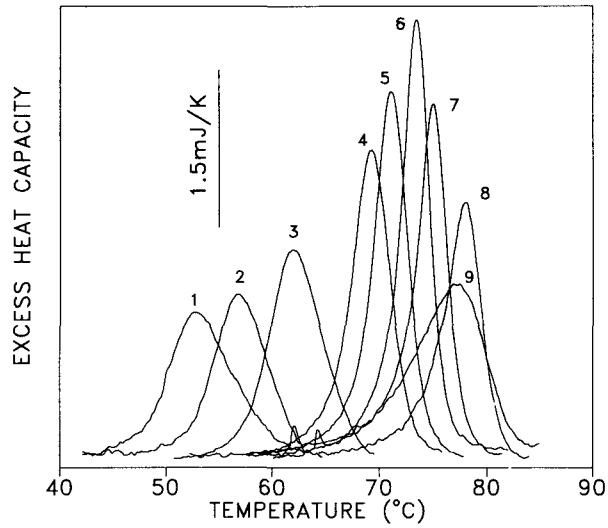


Figure 4. DSC scans of ferricytochrome c with ATP at various pH; *1* – 4.3; *2* – 4.62; *3* – 4.77; *4* – 5.20; *5* – 5.43; *6* – 5.86; *7* – 7.15; *8* – 8.26; *9* – 9.41. ATP concentration was 4 mmol/l, the protein concentration was 100–110 μmol/l. For buffers used see Materials and Methods.

concentration resulted in a decrease of r. If NaCl concentration exceeded 0.2 mol/l cytochrome c denatured as a monomer even in the presence of ATP. As is evident from Fig. 3 (inset) the effect of ionic strength on aggregation activity of ADP was stronger than that of ATP. ADP did not increase the aggregation of cytochrome c at NaCl concentration below 0.15 mol/l, and AMP had no effect on the aggregation of ferricytochrome c at any NaCl concentration tested.

As our previous results have shown, a more profound understanding of the properties of ferricytochrome c in the presence of ATP requires the characterization of its stability in a wider range of pH. This requirement stems from the fact that the coefficient of cooperativity of cytochrome c at pH 7.0 is higher than 1. As shown in Fig. 4, a change of pH affected significantly the shape and T_m of calorimetric curves of cytochrome c – ATP. The thermograms are symmetric (Fig. 4). The heating rate had no effect on the shape of the curves (not shown). The dependences of T_m on pH for cytochrome c and cytochrome c – ATP, ADP or AMP are shown in Fig. 5*a*. At pH 3.5–8.8 the T_m of cytochrome c was higher than that of cytochrome c – ATP. ADP only decreased the transition temperature within the pH range between 6 and 8, and AMP did not affect the transition of cytochrome c. An expressive increase in the transition temperature of cytochrome c was observed in all cases at pH 2–5 and at pH 5–10 there was only a little change of T_m. As seen in Fig. 5*b*, the calorimetric

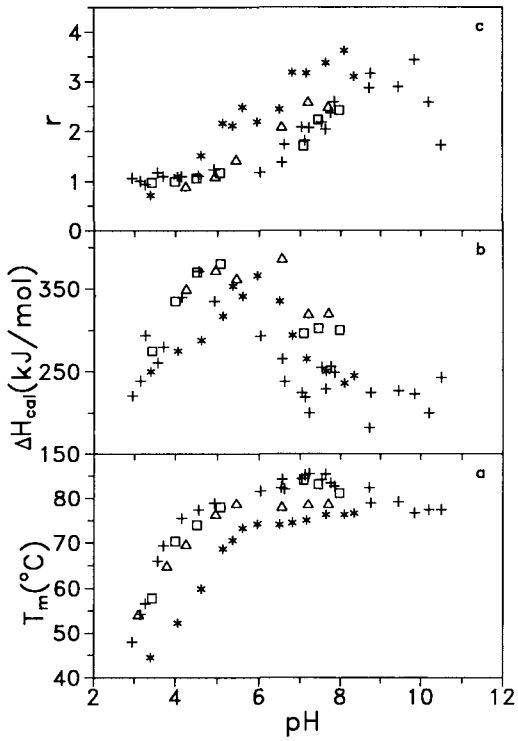


Figure 5. The dependences transition temperature T_m (5*a*), transition enthalpy ΔH_{cal} (5*b*) and cooperativity coefficient r (5*c*) on pH; (+) cytochrome c alone, (□) AMP, (△) ADP, (∗) ATP. The concentration of the nucleotides was 4 mmol/l, that of the protein was 100–110 μmol/l.

enthalpy of transition in acidic medium increased with the increasing pH, up to pH 5–6. At a higher pH (up to 8), ΔH_{cal} decreased. The maximum value of ΔH_{cal} for cytochrome c itself was 380 kJ/mol (pH 4.5), and in the presence of ATP it was 360 kJ/mol (pH 6). Consequently, the relationship between r ($\Delta H_{vH}/\Delta H_{cal}$) and pH (Fig. 5*c*), for both cytochrome c and cytochrome c – AMP, the value of r at pH 2.5–6.5 was close to 1. It is proposed that under these conditions the thermal denaturation is a two state process with no interaction between cytochrome c molecules. At pH higher than 7.0, r for cytochrome c gradually increased to reach 3.5 (at pH 8–10). At pH > 10 a decrease of r was observed. It is evident that ATP induced higher cooperation of cytochrome c which was associated with a shift towards lower pH values. The effect of ADP on ferricytochrome c cooperativity was significantly slighter than that of ATP.

The formation of ferricytochrome c aggregates was confirmed by gel chromatography at 25 °C. Fig. 6 shows the dependence volume of the eluent with the highest concentration of cytochrome c, on the pH media. It is evident that in phosphate buffers ferricytochrome c aggregation occurs at pH 7.5–8.5. An increase of ionic strength resulted in a decreased aggregation of ferricytochrome c. On the other hand, aggregation of ferricytochrome c occured at a lower pH (6–7) when

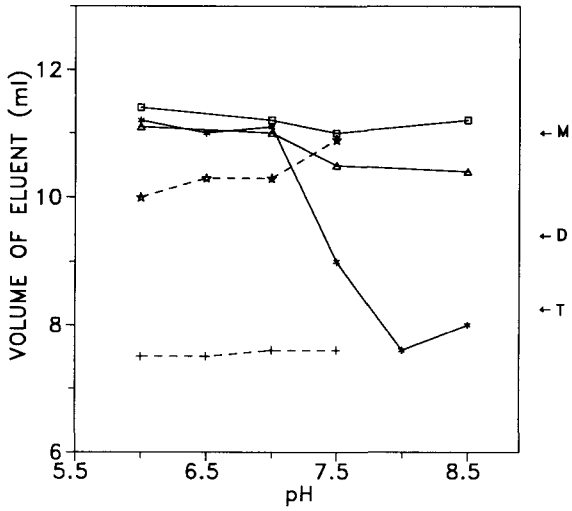
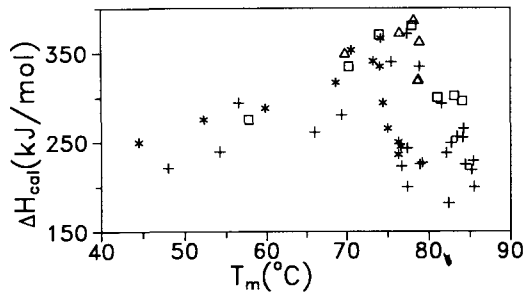


Figure 6. Gel chromatography of ferricytochrome c. The dependence of eluent volume with the highest concentration of the protein on pH. Sephadex G-75, 1 mg protein/0.2 ml, 2 mmol/l phosphate buffer. Dashed lines: (+) cytochrome c, 5 mmol/l ATP; (⋆) cytochrome c, 5 mmol/l ATP, 0.5 mol/l NaCl. Full lines: (*) cytochrome c; (△) cytochrome c, 0.1 mol/l NaCl; (□) cytochrome c, 0.5 mol/l NaCl. The arrows indicate the volume of eluent in which monomer M, dimer D or trimer T of ferricytochrome c were contained.

Figure 7. Dependence of calorimetric enthalpy of transition on temperature. For symbols and conditions, see legend to Figure 5.



ATP was added to low ionic strength media. Similarly as in the previous case, the ionic strength depressed the ATP effect on ferricytochrome c aggregation.

Calorimetric enthalpy of denaturation of small globular proteins has been shown to be a linear function of transition temperature (Privalov and Khechinashvili 1974). This dependence is limited to a narrow pH range. A change of the protein structure or a change in its mechanism would result in a change of this dependence. As shown in Fig. 7, there was an increase of ΔH_{cal} for cytochrome c alone or in the presence of AMP and ADP within a temperature range from 35 to

75 °C; this increase can be described by:

$$\Delta H_{\mathrm{cal}} = 5.8\,T_{m,\mathrm{K}} - 1645.5 \quad (\mathrm{c.c.} = 0.96)$$

The slope of this line expresses the change of the heat capacity for thermal transition (ΔC_p), and its value 5.8 kJ/mol.K corresponds with the published values of 5.76 kJ/mol.K (Kuroda et al. 1992) and 5.0 kJ/mol.K (Potekhin and Pfeil 1989). This linear dependence holds for pH 2.5–4.5. A similar linear relationship exists for the system cytochrome c plus ATP, which may be described by the equation:

$$\Delta H_{\mathrm{cal}} = 3.8\,T_{m,K} - 967.9 \quad (\mathrm{c.c.} = 0.95)$$

The lower change of heat capacity may be connected with the fact that, at a lower pH, ATP supports the formation of molten globule state of ferricytochrome c (Goto et al. 1991).

A rather interesting dependence of ΔH_{cal} on T_m could be observed at pH 4.5–7.0. As shown in Fig. 7, an increase in transition temperature over 75 °C resulted in a decrease of ΔH_{cal} for cytochrome c as well as for cytochrome c with nucleotides. These dependences could be approximated by the equations:

$$\Delta H_{\mathrm{cal}} = -18.1\,T_{m,\mathrm{K}} + 6709 \quad (\mathrm{c.c} = 0.94), \text{ for ferricytochrome c,}$$

$$\Delta H_{\mathrm{cal}} = -17.8\,T_{m,\mathrm{K}} + 6568 \quad (\mathrm{c.c.} = 0.93), \text{ for ferricytochrome c with AMP, and ADP}$$

$$\Delta H_{\mathrm{cal}} = -37.7\,T_{m,\mathrm{K}} + 13430 \quad (\mathrm{c.c.} = 0.81), \text{ for ferricytochrome c with ATP.}$$

While in the presence of nucleotides this dependence may be affected by the association of cytochrome c molecules, for cytochrome c alone at pH 4.5–6.5, the two state process without protein intermolecular cooperation is characteristic, and the observed negative value of slope refers to a change in the mechanism of cytochrome c denaturation. Since in this pH range the reversibility of thermal denaturation is only 50–80%, the question arises concerning the observed negative slope. At pH exceeding 7.5 there was only a slight rise in ΔH_{cal} with the decreasing temperature, and it can be predicted that in this medium further changes in the thermal transition mechanism of cytochrome c may occur.

Previous microcalorimetric ligand – protein binding studies have shown when ligand is bound to the denaturation state of the protein the transition temperature decreases (Edge et al. 1985; Shrake and Ross 1992). As shown in Fig. 1, there was a significant decrease in T_m of ferricytochrome c with the increasing concentration of ATP. A similar effect is evoked by the binding of protons to ferricytochrome c (Fig. 5). The general scheme of denaturation in this case can be represented as

$$N + hH^+ \Leftrightarrow DH_h^+$$

where N is the native and D the denatured state of the protein, and h is the number of protons bound upon denaturation. The equilibrium constant K of the reaction can be expressed as

$$K = \frac{[DH_h^+]}{[N]\,[H^+]^h}$$

where the members in the brackets stand for concentrations of the reaction partners (we assume the activities to be equal to the concentrations). From the assumptions that a) the dissociation constant of proton does not differ much from that of the protein, b) the denaturation enthalpy of ferricytochrome c does not depend on pH, c) the change of ΔC_p does not depend on the temperature; an equation may be constructed for the relationship between pH and transition temperature:

$$2.303\,pH = -\frac{1}{\mathrm{R}h}\left(\frac{a}{T_{m,\mathrm{K}}} - \Delta C_p \ln T_{m,\mathrm{K}}\right) - \frac{\Delta C_p}{\mathrm{R}h}\,(1 + \ln T_0)$$

where R is the gas constant, a is the enthalpy of thermal transition extrapolated to 0 K, and T_0 is the temperature at which the transition entropy drops to zero (Potekhin and Pfeil 1989). In practice, the above assumption can be met when the slope of the dependence ΔH_{cal} vs $T_{m,\mathrm{K}}$ of transition enthalpy on the transition temperature has a value equal to the observed transition heat capacity change of the protein. Within pH 2.5–4.5, a good agreement was observed between the values (ΔC_p from the thermograms ranged between 4 and 6 kJ/mol.K, and that from the slope was 5.8 kJ/mol.K). As shown in Fig. 8*A*, there is a linear relationship between the solution pH and the parameter $(a/T_{m,\mathrm{K}} - \Delta C_p \ln T_{m,\mathrm{K}})$. From the slope in the linear region a value of $h = 3.3$ can be obtained. It means that 3.3 protons are bound to the denatured state of ferricytochrome c during thermal transition. This value is slightly smaller than that observed by Potekhin and Pfeil (1989). We suppose that this difference may be due to a lower concentration of buffer, or a wider region at higher pH used in our experiments. It is evident from Fig. 8*A* that at pH higher than 4 the slope increases consequently, the number of binding protons decreases. A similar change in h has been observed in other globular proteins around neutral pH of the media (Privalov et al. 1971).

As it is evident from Fig. 5*c*, ATP induced aggregation of ferricytochrome c even at acidic pH of the media. As shown in Fig. 4, ΔC_p read from the thermograms was virtually zero. However, difficulties with posttransition baselines limit the accuracy of this value, and generally it is considered to be more reliable to estimate ΔC_p by taking the slope of the ΔH_{cal} vs T_m plot ($\Delta C_p = 3.3$ kJ/mol.K). As shown in Fig. 8*B*, linear dependence was observed between pH and parameter $(a/T_{m,\mathrm{K}} - \Delta C_p \ln T_{m,\mathrm{K}})$. It could be estimated from the slope of this function that 1.7 protons bound to one molecule of ferricytochrome c at denatured state during thermal transition. In this case, the reaction scheme will be modified:

$$1/3\,N_3ATP_x + h'H^+ \Leftrightarrow 1/3\,D_3ATP_yH_{h'}^+$$

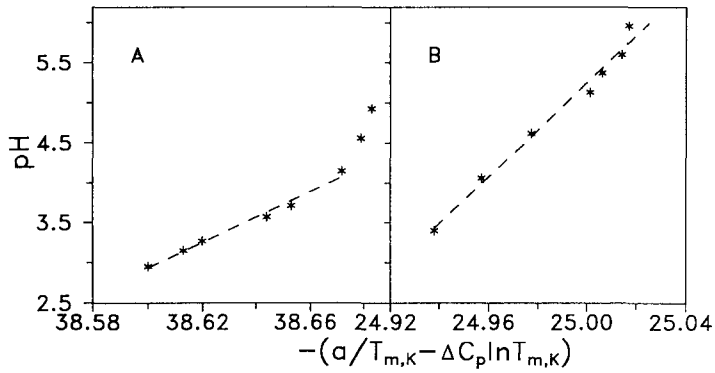


Figure 8. The dependence of the pH value of the medium at which transition occurs, as a function of thermodynamic parameters for transition $(a/T_{m,\mathrm{K}} - \Delta C_p \ln T_{m,\mathrm{K}})$; 2 mmol/l buffers. *A*) without ATP; *B*) 4 mmol/l ATP. The least-square analysis gave $h = 3.3$ for samples without ATP, and $h = 1.7$ for samples with ATP.

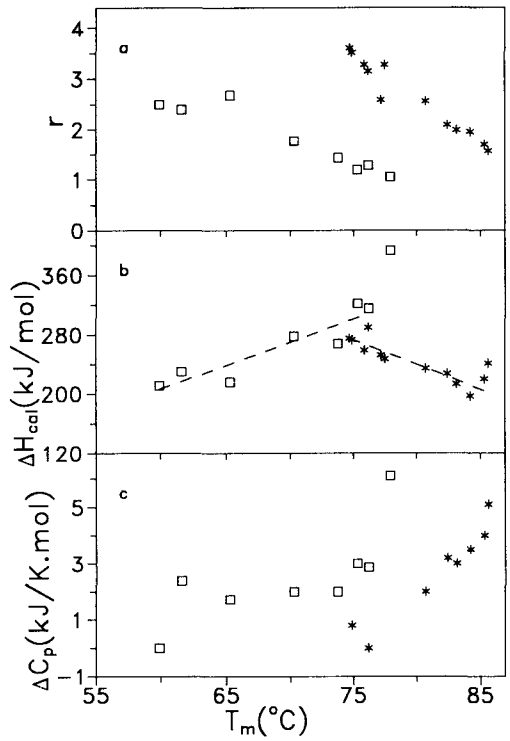


Figure 9. The dependence of thermodynamic parameters of transition ferricytochrome c on temperature transition induced by a change of ATP concentration. The concentration of ATP was varied from 0 to 5 mmol/l. (∗) 2 mmol/l phosphate buffer, pH 7.0; (□) 2 mmol/l acetate buffer, pH 4.5. The dashed line was constructed by the least-square analysis, and it represents the equations: $\Delta H_{\mathrm{cal}} = 6.3\,T_m - 170$, for pH 4.5, and $\Delta H_{\mathrm{cal}} = -6.7\,T_m + 777$, for pH 7.0.

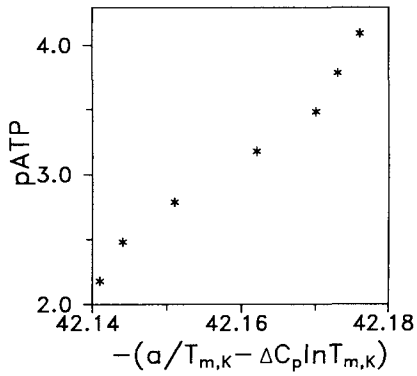


Figure 10. The dependence of pATP (–log[ATP]) at which transition takes place, as a function of the thermodynamic parameters of transition ($a/T_{m,\mathrm{K}} - \Delta C_p \ln T_{m,\mathrm{K}}$) at pH 4.5.

as the coefficient of cooperativity r is close to 3, where x and y are unknown figures of ATP numbers bound to N or D states of ferricytochrome c. From this analysis it is not clear whether the smaller number of binding protons h' is a result of ATP binding or of the aggregation of ferricytochrome c.

Fig. 9*a* shows the dependence of r on the transition temperature induced by a change of ATP concentration at pH 4.5 or 7.0. At both these pH values the coefficient of cooperativity r increased with the decrease of T_m. On the other hand, the slopes of the linear dependences ΔH_{cal} (Fig. 9*b*) were 6.2 and −6.7 for pH 4.5 and pH 7.0 respectively. In addition, as follows from Fig. 9*c*, the denaturation heat capacity change of the protein drops to zero with the decreasing temperature (saturated ATP concentration). Similarly as with pH – induced changes of T_m, there are difficulties with the posttransition baseline that limit the accuracy of ΔC_p value obtained directly from the thermogram. Using the slope value from the linear function of ΔH_{cal} vs T_m at pH 4.5, $\Delta C_p = 6.2$ kJ/mol.K, (Fig. 9*b*) the dependence of pATP (-log[ATP]) on parameter ($a/T_{m,\mathrm{K}} - \Delta C_p \ln T_{m,\mathrm{K}}$) was constructed, similarly as in the case of H^+ binding. As shown in Fig. 10, this dependence is nonlinear upon subdividing this dependence into three parts (monomer, dimer and trimer of ferricytochrome c) and the slope was calculated for each of them, the following ratios were observed: for ATP concentrations between 0.08 and 0.4 mmol/l, one molecule of ATP bound to the denatured state of the protein; between 0.4 and 1.5 mmol/l ATP 1.5 molecule, and between 1.5 and 5.0 mmol/l one molecule of ATP bound to ferricytochrome c. For lower ATP concentrations (0–0.5 mmol/l) the values of ATP bound to denatured protein may be underestimated because the added ATP concentrations were close to the concentration of the protein. Conformational transition according to the general scheme (trimer of ferricytochrome c) occurs at pH around 4.5, and the saturated concentration of ATP (5 mmol/l) can be presented as

$$1/3\, N_3 ATP_x + 1.7\, H^+ + 1.0\, ATP \Leftrightarrow 1/3\, D_3 ATP_{x+1} H^+_{1\,7}$$

While estimation of the numbers of protons and ATP bound to ferricytochrome c during thermal transition was possible for pH values between 2.5 and 5.0, there was a negative value for the slope dependences of the calorimetric enthalpy of transition on transition temperature for pH values exceeding 5 (Fig. 7 and 9*b*); thus the numbers of bound protons and ATP was not estimated. Probably, the change in enthalpy of transition in dependence on pH or ATP concentration cannot be neglected in this case. The meaning of the linear dependence of calorimetric enthalpy of thermal transition on transition temperature is rather vague, and probably it is connected with the processes of hydration of polar groups during thermal transition (Makhathadze and Privalov 1993).

Discussion

Triphosphate groups play a crucial role in the energy metabolism. In spite of the fact that all nucleosides are basic carriers for triphosphate, at the inner mitochondrial membrane virtually all processes of triphosphates synthesis occur on adenosine. However, the regulation of primary energy metabolism must be sensitive to the specific triphosphate group. As shown in Table 1, the triphosphates studied present rather similar concentration ranges of aggregation activity on ferricytochrome c. ADP has a weaker aggregation effect at higher concentrations, and AMP has no aggregation activity at all. The difference between the efficiency of ATP and ADP increases at higher ionic strengths (0.15 mol/l NaCl) where no ferricytochrome c aggregation by diphosphate is observed. On the other hand, some differences may be seen in relation to the base in triphosphates.

The cytochrome c protein chains in eukaryotic cells contain a higher number of lysine, arginine and histidine residues. At pH 2, horse heart cytochrome c can carry up to 24 positive charges in the protein chain. The electrostatic repulsion of charges depresses the creation of a compact structure in the acidic medium. At this pH, cytochrome c shows no thermal transition. Formation of a tighter structure may be achieved by blocking the positive charges. Sufficient concentration of anions results in the formation of molten globule state of cytochrome c. While chloride anions induce the occurrence of this state even at 0.5 mol/l, anions with a higher number of negative groups are more effective (Potekhin and Pfeil 1989; Kuroda et al. 1992). As is shown by Goto et al. (1991), ATP is effective at concentrations below 5 mmol/l. Characterization of molten globule state at high ionic strength (pH from 2.2 to 2.9) showed that the transition temperature of ferricytochrome c was significantly higher than that at low ionic strength at the same pH. The enthalpy of transition for molten globule state is linearly dependent on the transition temperature, and the slope of this dependence has an expressively lower value when compared with that at low ionic strength (Potekhin and Pfeil 1989; Kuroda et al. 1992). A reason for the observed difference is not sufficiently recognized. ATP induces the molten globule

state of cytochrome c at a lower concentration than does NaCl and therefore, it would be very interesting to characterize the thermal behavior of cytochrome c in these conditions. Unfortunately, in media below pH 3.5 and using 100 μmol/l cytochrome c (concentration suitable for calorimetric measurements), aggregation of the protein occurs even at lower temperatures (4 °C), and no investigation by DSC of the molten globule state of cytochrome c in the presence of ATP is possible.

Another way to decrease the number of positive charges in the protein chain is dissociation of the proton from cytochrome c amino acid groups. At pH ranging between 2 and 7, protons dissociate from carboxy groups which acquire negative charges; in the case of histidine, a neutral group is formed. In a pH range between 2 and 4 structural changes occur near the heme and also in the protein chain, which at higher pH gives rise to state III (Theorell and Åkesson 1941; Myer and Saturno 1991). As shown in Fig. 5, upon increasing pH also transition enthalpy increased. The slope of this linear relationship was significantly higher than that for the molten globule state of ferricytochrome c. During thermal unfolding of the protein the prevalent contribution of enthalpy comes from the hydration of the hydrophobic part (Makhatadze and Privalov 1993). A comparison of these dependences for different buffer concentrations in the range of 40 mmol/l glycine/HCl (Privalov and Khechinashvili 1974), or 15 mmol/l glycine/HCl, 15 mmol/l acetate (Fu and Freire 1992) with those used in our experiments (2 mmol/l glycine/HCl and 2 mmol/l acetate) gave similar slopes and transition temperatures for the same pH values. These results indicate that the interaction between the groups of cytochrome c and these buffers have but a small effect. Despite the fact that ATP induced association of the protein molecules at pH ranging between 3.5 and 5.8 ($r \neq 1$), the transition enthalpies are similar for equal temperatures. The lower value of the slope enthalpy dependence on temperature is due to a higher enthalpy at a lower temperature which may be related to the formation of molten globule state of cytochrome c in the presence of ATP. It can be assumed that ATP does not change the structure of cytochrome c in these media.

At a higher pH, a significant change in the behavior of cytochrome c occurs as compared with pH range 2.5–4.5. As shown in Fig. 5, the transition enthalpy at pH 4.5–7.5 decreases gradually while the transition temperature slightly increases. No great changes were observed in the structure of native cytochrome c upon increasing pH from 4 to 8 (Wooten et al. 1981). In this range of pH the dissociation of probably two protons, one from propionic acid and another from histidine (pK 5.2 and 6.4, resp.), occurs (Shaw and Hartzell 1976; Marini et al. 1981). Some authors have assumed dissociation of propionic acid outside this range (Harstshorn and Moore 1989). From the thermal changes of electron absorption spectra and CD measurements it follows that at this pH range cytochrome c has two transitions (Myer 1968). The first one occurs at a lower temperature near the heme, and the second one changes the structure of the whole protein. In our conditions,

the first transition was not observed by DSC. However, at higher concentrations of HEPES (0.05–0.5 mol/l) an apparent thermal transition similar to that observed by Muga et al. (1991) or Santucci et al. (1989) was followed. From the concentration dependences of buffers and the protein it is evident that at the beginning of thermal transition, aggregation of cytochrome c occurs as an exopeak (not shown). Therefore, at this range of pH it is necessary to find the best conditions in which aggregation does not occur in the form of an exopeak. In our study, a very low concentration of buffer was used, and as shown in Fig. 5, at pH higher than 7.5 intermolecular interaction of cytochrome c occurs as a very high ratio between van't Hoff and calorimetric enthalpy. On the other hand, upon changing the pH between 7 and 10, even at a very low concentration of buffers, a relatively high dispersion of ΔH_{cal} was observed at the same pH (Fig. 5). Despite these experimental problems within pH 4.5–7.0, the linear function of ΔH_{cal} on T_m, has a negative slope. Since for this range r is still close to 1, it can be assumed that there is apparently a two state process of denaturation without any interaction of the protein molecules. The decrease in ΔH_{cal}, however, may be connected with the spectroscopically observed transition near the heme. Another way to explain the behavior of cytochrome c may be a pH – dependent change in the properties of denatured cytochrome c. As shown by kinetic measurements of folding guanidine chloride denatured cytochrome c, the reaction rate changes within a pH range of 4–7 (Nall et al. 1988). Further measurements are required for a better characterization of the thermal transition in this pH range.

An important observation concerning ATP action on cytochrome c is that the function enthalpy on the transition temperature is very similar in both cases. One can assume that ATP may not essentially affect the structure of cytochrome c, and may not change the mechanism of denaturation. A weak effect of ATP on the tertiary structure of cytochrome c at room temperature supports the results of a NMR study of the interaction of polyphosphate with cytochrome c (Whitford et al. 1991). Similarly, as in the NMR study, from the DSC study it is evident that there is a significantly higher association of cytochrome c with triphosphate moiety. In the pH region around 7.0 at the ionic strength of about 0.15 mol/l, protein aggregation can be viewed as a result of favourable interactions with the help of negatively charged triphosphates. The formation of bridges between molecules of ferricytochrome c has been shown by Concar et al. (1991). The specific sites of polyphosphate binding are close to lysine 13, 86 and 87. These positive groups on the surface of ferricytochrome c also participate in the formation of complexes of ferricytochrome c with its own redox partners (Osheroff et al. 1980; Stonehuerner et al. 1985). Therefore, it is likely that the inhibition of electron transfer from cytochrome c reductase to cytochrome c oxidase by ATP is caused by the suppression of the effective binding of cytochrome c on their redox partners. In our study, we also observed that associates between molecules of cytochrome c can be formed

during the interaction of this protein with heparin as a model for redox partners of cytochrome c (Bágeľova et al 1994)

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