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Biophysics of *T5*, *IRA* phages, *Escherichia coli* outer membrane protein *FhuA* and *T5-FhuA* interaction

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Abstract In spite of the similarities in a structural organization of *T5* and *IRA* phages their thermal and hydrodynamical peculiarities are completely different. One of the significant differences is observed in temperature value at which thermally induced DNA ejection starts. If in the case of physiological conditions this difference equals to 30°C, then it decreases as ionic strength of the solvent decreases. Also, from our experimental results follows that in the opening of phage tail channel for *T5* phage (at *pH7*) significant role-play electrostatic forces. In spite of that both of these phages grow on the same *Escherichia coli* strain, we have shown that these phages need different receptors to penetrate into the bacterial cell precisely *FhuA* serves as receptor only for *T5* phage. The higher *FhuA* concentration in *T5* phage suspension is, the more intensive DNA ejection in environment is. The minimal *FhuA*/*T5* ratio, which is 300/1, correspondingly, necessary for effective DNA ejection from the phage head was experimentally determined. For the first time the ejection of *T5* phage DNA induced by *FhuA* was observed in an incessant regime. The deconvolution of calorimetric curve of *FhuA's* denaturation has been shown that in a chosen condition there are four thermodynamically independent domains in the structure of *FhuA*.

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Abbreviations DASM-4A: Differential adiabatic scanning microcalorimeter · LB: Luria broth · LDAO: Lauryldimethylaminoxide · Octyl-Poen: Octyl-oligo-oxyethylene · PBS: Phosphate-buffered saline · FPLC: Fast protein liquid chromatography

Introduction

In terms of human morbidity and mortality, bacterial disease continues to be of pressing concern. While standard treatments of many bacterial infections involve an application of chemical antimicrobials such as antibiotics, in fact bacterial viruses, known as phages, can display comparable antibacterial efficacy. To better understand the phage infection process giving rise to phage antibacterial activity, we have focused on the initial stages of infection—which involves cell-surface recognition, attachment, and viral genome ejection into the cytoplasm—since disruption of phage infection is considerably easier prior to genome ejection. Here we employ various methods to determine the conditions required for DNA ejection from phage particles and how this ejection is influenced by physico-chemical factors. To have a clear idea of the mechanism of phage binding, it is advisable to investigate the properties of both the interacting objects, phage and phage receptor. Here we consider the thermal and hydrodynamical properties of these objects. By denaturing virion particles under the influence of different factors, for example, one can obtain information about multi-domain structures and stability. We have studied in particular the thermodynamics of protein stability by obtaining precise calorimetric measurements during temperature-induced protein denaturation (Privalov and Potekchin 1986; Privalov and Khechinashvili 1974; Privalov 1982). Calorimetry also allows reliable evaluation of thermally induced DNA ejection from phage particles (Mdzinarashvili et al. 2000a, 2001; Mrevlishvili et al. 1990, 2001). The mechanism of DNA molecule transition from a

packed state inside a phage head to an unpacked, random-coil state in the phage environment (DNA ejection) may be determined by viscometry. The premise behind viscometry is that the viscosity of phage suspensions is indistinguishable from the viscosity of the solvent alone, whereas at phage DNA ejection viscosity sharply increases (Mdzinarashvili et al. 2000a, b, 2001).

By means of viscometer (Mdzinarashvili et al. 2001), which allows observation of the kinetics of thermally induced release of DNA from the phage particle; the strength of interactions between DNA and capsid inside of the phage can be characterized. In the present work it is shown that these electrostatic forces should play an important role in phage DNA–protein interactions.

There are specific receptors on the surface of bacterial cells, by which phage recognize their host cell, but the information about these initial phage-bacterial contacts is extremely limited. Phages possessing icosahedral symmetry of the head have short "thorns"(or hooks) that they employ for cell attachment over short distances, and long, hinged fibers that they employ for phage adsorption over long distances. It can be supposed for phage adsorbing to a cell surface that the nature of these contacts should be different. To study these contacts it is necessary to identify factors that influence the strength of their interaction with host receptor molecules. This means that there will be a possibility to make an influence on these contacts and if it will be necessary interrupt them, i.e. cessate bacterial infection by phage at initial stages. We employ a model system consisting of phage and bacterial outer membrane protein (the phage receptor). Such a phage–receptor combination is phage T5 and its receptor, FhuA, which is detected by means of light scattering (Frutos et al. 2005), fluorescence spectroscopy (Boulanger et al. 1996; Plancon et al. 1997), and cryo-electron microscopy (Plancon et al. 2002). Both

fluorescence experiments and cryo-electron microscopy images show that DNA release from T5 phage can be triggered simply by interaction of the virus with its purified receptor. It is also

known that T5 phage uses a two-step mechanism for transfer of its DNA into host cells. After attachment of phage T5 to its FhuA receptor, 8% of the chromosome is first injected, and then there is a pause during which proteins encoded by this DNA fragment are synthesized, allowing the remaining DNA to be injected (Boulanger and Letellier 1992). Other phages, such as Un, DDVI, Sd, also possess the feature of multistep ejection of DNA, but in the case of thermally induced ejection. (Mdzinarashvili et al. 2001; Mrevlishvili et al. 1999, 2001; Khvedelidze et al. 2004).

In the present paper we show that a thermally induced, multistep ejection of DNA from both T5 and IRA phages can be observed by means of viscometry. Ferguson et al. (1998) and Locher et al. (1998) provide models of FhuA structure and function. In our case by "function" and "structure" we mean the FhuA structure as it is found in the membrane, i.e. with close contact between protein and both polar and nonpolar molecules (the contacts of protein with water and with membrane phospholipids, respectively). A two-domain model of FhuA structure in the bacterial membrane has been proposed (Bonhivers et al. 2001; Ferguson et al. 1998; Locher et al. 1998). However, some doubts are cast upon two-domain structure of FhuA, consisting of 714 amino acids (Bonhivers et al. 2001; Boulanger et al. 1996) versus an alternative function structure of >2 domains that may exist under certain conditions (http://bio-p.ox.ac.uk/www/lj2000/sansom/sansom_06.html 1999). We suppose that the two-domain structure of FhuA limits the protein's function, whereas the presence of more than two domains could allow multiple functionality.

Materials and methods

The IRA and T5 phages, and *Escherichia coli* outer membrane protein FhuA, receptor for T5, were chosen as objects of investigation. T5 and IRA phages belong to the same morphological group of phages with icosahedral symmetry of phage head and long noncontractile tail. Based on electron-microscopy data the sizes of these phages are following: T5 phage head sizes are 750 Å×750 Å, the tail sizes are 1,800 Å×120 Å; IRA phage head sizes are 500 Å×500 Å, the tail sizes are 500 Å×500 Å (Adamia et al. 1990). Both of the phages contain one ds-DNA molecule with molecular weight for T5 phage –80×106 Da, and for IRA phage –84×106 Da (Adamia et al. 1990). The growth of phages was carried out on Freser fermenter at 37°C. The purification was carried out by centrifugation in a CsCl density gradient and the concentration of phages was determined via its DNA using a spectrophotometer, assuming that absorption of 0.023 at the wavelength of 260 nm corresponds to 1 μg/ml. The concentration of DNA was calculated taking into account the DNA-to-phage protein ratio. For T5 phage the DNA makes up 70% of the phage particle with the other 30% consisting of protein (Frutos et al. 2005). Correspondingly, the IRA phage is 43% DNA and 57% protein (Adamia et al. 1990).

The *E. coli* outer membrane protein, FhuA, was purified at the Department of Membranes and Receptors, ESBS, UPR, Strasbourg, France. The *E. coli* K12 HO830 strain, transformed by the PHX405 plasmid and overproducing the FhuA protein, was used for this purification. Hexahistidine-tagged FhuA protein was purified as previously described (Moeck et al. 1996; Lambert et al. 1999). Lauryldimethylaminoxide (LDAO) was used for the solubilisation and purification of the protein. The pooled fractions from the nickel-chelating column (HiTrap HP Amersham Pharmacia) were further purified on an anion exchange column (Mono-Q HR 5/5 Amersham Pharmacia) using buffer Tris 20 mM pH 8.0 LDAO 0.05% w/vol. The protein was eluted with a 0–50% NaCl 1 M gradient.

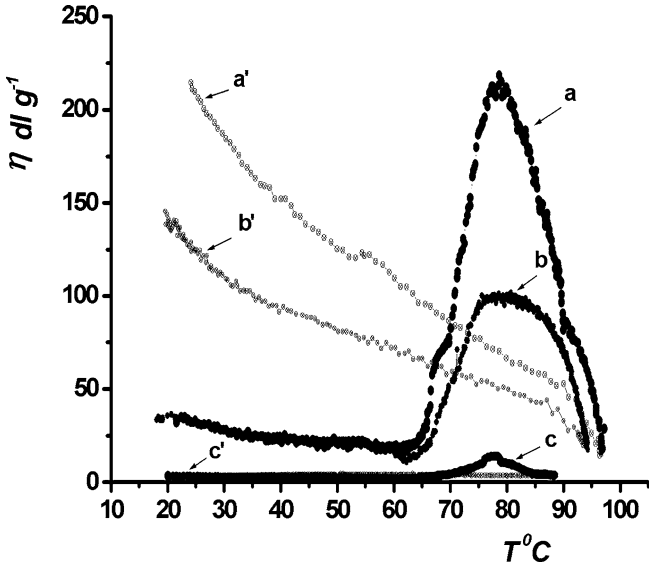

Fig. 1 Dependence of the specific viscosity of *IRA* phage suspension on temperature at various ionic strength of the solvent. **a** 0.15 M NaCl + 0.05 M phosphate buffer, pH 7; **b** 0.015 M NaCl + 0.05 M phosphate buffer, pH 7; **c** 0.5 M NaCl + 0.05 M phosphate buffer, pH 7. **a′**, **b′**, **c′** curves correspond to the phage DNA renaturation process

Under these conditions protein FhuA may be stored for several months at 4°C, during which time activity does not dramatically decline. Protein concentration was measured spectrophotometrically, considering that at 280 nm 1.2 OD corresponds to 1 mg/ml. Before experiments the FhuA solution was centrifuged at 2,800*g* for 20 min and then 1 ml of the sample was dialyzed versus 300 ml of buffer containing 0.15 M NaCl, 0.05 M phosphate buffer, 0.03% LDAO, pH 7.05. The solvent used for dialysis was also applied both in calorimetric and spectrophotometric measurements. The thermal denaturation of FhuA and phages was conducted using a differential scanning microcalorimeter DASM-4A (Russia). The spectrophotometric measurements of FhuA were done by means of spectrophotometer SPECORD–40 M UV (Germany, Jena). Hydrodynamic properties of the objects of investigation separately and also in complex (phage-FhuA complex) were studied by means of Zimm-Crothers type viscometer constructed in our laboratory, with automated logging of rotations.

Results

The dependence of specific viscosity of IRA phage on temperature at various solvent ionic strengths is given in Fig. 1. As it is evident from the figure, the initial temperature of DNA ejection does not significantly shift with increasing solvent ionic strength, but the maximal degree of viscosity changes do. The higher the ionic strength, the higher the maximal viscosity following phage-DNA ejection (Fig. 1). This result allows us to suppose that the degree of DNA release from phage heads depends on the ionic strength of the solvent, with greater solvent ionic strength resulting greater DNA release. Based on experimentally determined dependence of specific viscosity of DNA on its molecular weight (Freifelder 1976), it may be assumed that maximal value of viscosity of IRA phage DNA is Mr 84 MDa after DNA ejection into the solvent has to be more than 200 dl/g (approximately two times higher) (Freifelder 1976).

The dependence of the specific viscosity of T5 phage suspensions on temperature, at various solvent ionic strengths, is given in Fig. 2. The curve of thermally induced DNA ejection from T5 phage at low (as compared with physiological conditions) solvent ionic strength is shown on Fig. 2a, and the curve of DNA ejection at physiological ionic strength is given on Fig. 2b. As it is evident from the figure, at physiological conditions (Fig. 2b) the curve is presented by two regions of viscosity increase, which point to DNA ejection occurring in two stages. The first stage occurs over a temperature range of 30–45°C while the second stage occurs over a temperature range of 63–68°C. In comparison with this curve under physiological conditions, the curve at low solvent ionic strength (Fig. 2a) presents only one range over which DNA ejection occurs, from 55°C to 64°C. That result points to DNA ejection occurring over only a single step at low solvent ionic strength.

There are some differences in these results with IRA phage under the same conditions (Figs. 1, 2). If we compare both curves b in Fig. 1 (for IRA phage) and Fig. 2 (for T5 phage) we see that the value of the specific viscosity for T5 phage is up to 350 dl/g while for IRA phage is approximately 100 dl/g under the same conditions. Analogously, comparing curves c (Fig. 1) and a (Fig. 2), it is clearly seen that the maximal value of specific viscosity for T5 phage reaches almost 500 dl/g while that for the IRA phage under the same conditions

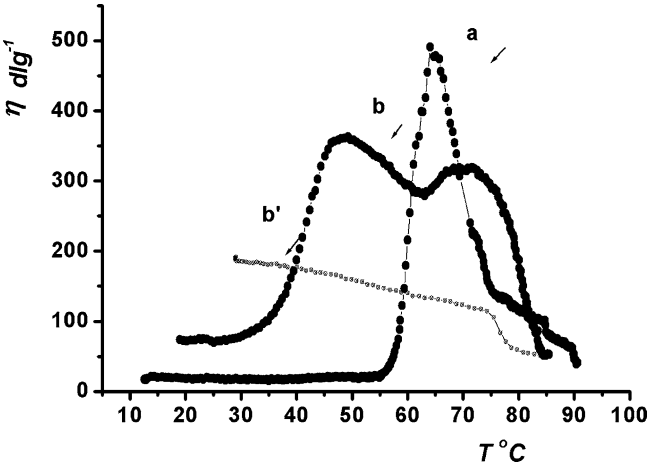

Fig. 2 Dependence of the specific viscosity of *T5* phage suspension on temperature at various ionic strength of the solvent. **a** 0.15 M NaCl + 0.05 M phosphate buffer, pH 7; **b** 0.015 M NaCl + 0.05 M phosphate buffer, pH 7. **b′** curve corresponds to the phage DNA renaturation process

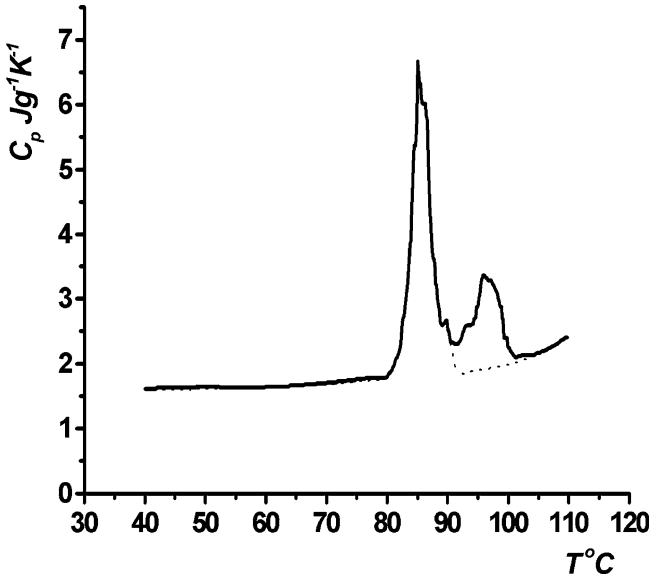

Fig. 3 **a** Dependence of the specific heat capacity of T5 phage on temperature. **a′** Dependence of the specific heat capacity of T5 phage on temperature at reheating

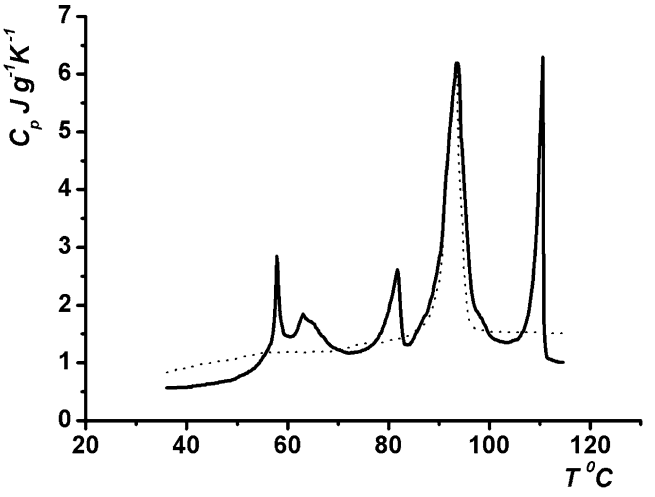

Fig. 4 **a** Dependence of the specific heat capacity of IRA phage on temperature. **a′** Dependence of the specific heat capacity of IRA phage on temperature at reheating

is only up to 20 dl/g. We may conclude from these results that with decreasing solvent ionic strength the maximal value of specific viscosity for T5 phage increases while that for IRA phage decreases. Besides, it should be mentioned that the DNA ejection for T5 phage in comparison with IRA phage significantly depends on variation of ionic strength of the solvent (Figs. 1, 2).

As noted in the introduction, while the T5 and IRA phages have a similar morphological organization, they are distinguishable by their hydrodynamic properties (i.e., DNA ejection as measured by solvent viscosity) and thermodynamic (i.e. calorimetric) features. The dependence of specific heat capacity of T5 phage on temperature is given in Fig. 3a (at physiological conditions of the solvent). The recorded curve is presented by two heat absorption peaks. To determine what phage component these peaks correspond to (DNA vs. protein), we have carried out reheating of the phage solution after cooling the solution of denaturated phages in a scanning regime (Fig. 3b). The result of this reheating allowed us to determine that the heat absorption peak at a temperature interval of 80–93°C corresponds to T5 phage DNA denaturation, and hence that the next peak (93–103°C) corresponds to T5 phage protein denaturation. Based on viscometric data for T5 phage (Fig. 2b), DNA ejection occurs over a temperature range of 30–45°C and then between 63°C and 68°C. As there is no heat effect on the calorimetric curve of T5 phage over this range (i.e., no peak or depression; Fig. 3), we infer that DNA ejection from the phage particle is a non-enthalpic process.

The result for IRA phage is completely different from that observed for T5 phage, though experiments were carried out under the same conditions [PBS (0.15 M NaCl + 0.05 M phosphate buffer), rNN 7]. The dependence of specific heat capacity of IRA phage on temperature is given on Fig. 4. The curve is presented by several heat absorption peaks over a large temperature range (from 55°C to 112°C). Both the result of reheating of IRA phage solution and of viscometric data allowed us to determine the temperature area (85–95°C) in which the phage DNA denaturates. Therefore, the other heat absorption peaks during the additional temperature intervals (55–70°C, 75–85°C and 105–112°C) must correspond to denaturation of the protein capsid.

We also investigated the influence of ionic strength on thermodynamical properties of phages T5 and IRA. It has been shown that variation in solvent ionic strength influences only T5 phage. The dependence of specific heat capacity of T5 phage on temperature in the case of low ionic strength of the solvent is shown on Fig. 5. The result of reheating the phage solution shows that the DNA does not completely renature its structure under these conditions. It should be mentioned that low solvent ionic strength did not influence IRA phage DNA renaturation (Fig. 6).

The attention should be paid to that the calorimetric study of these phages (Figs. 5, 6) in the case of low ionic strength of the solvent (as well as in physiological conditions) show the DNA ejection as nonenthalpy process.

We have also studied thermal properties of *E. coli* outer membrane protein-receptor, FhuA, by means of calorimetry. The calorimetric result of heat denaturation of FhuA is given in Fig. 7 (solid line). Two heat-absorption transitions point to the complexity of the protein denaturation process, which is not surprising considering that two ordered structures form, one in its polar part and one in its nonpolar part (summarized, specific enthalpy of denaturation is equal to 2,200 ± 200 kJ/mol). Such a big specific enthalpy value directly indicates that the protein in a chosen by us conditions has high ordered structure. Two transitions

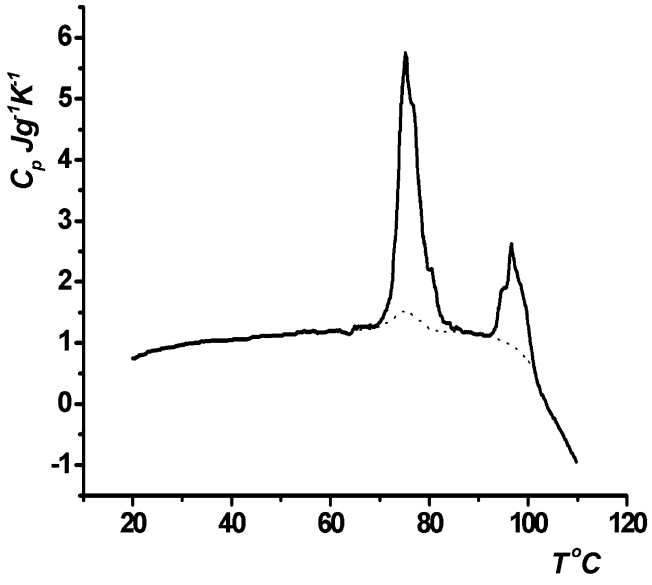

Fig. 5 **a** Dependence of the specific heat capacity of T5 phage on temperature in the case of low ionic strength of the solvent. **a′** Dependence of the specific heat capacity of T5 phage on temperature at reheating

allows make a conclusion, that the denaturation process of FhuA thermodynamically is not a transition between two stable states, but there is even if one more intermediate thermodynamic stable state (Privalov and Potekchin 1986). It means that while temperature is increased the membrane protein passes from the native state through a number of intermediate macro-states until finally reaching a denatured state.

We have carried out the deconvolution of recorded calorimetric curve of FhuA (Fig. 7; dotted line). Four peaks were obtained as the result of deconvolution and each peak is elementary, considering that these transi-

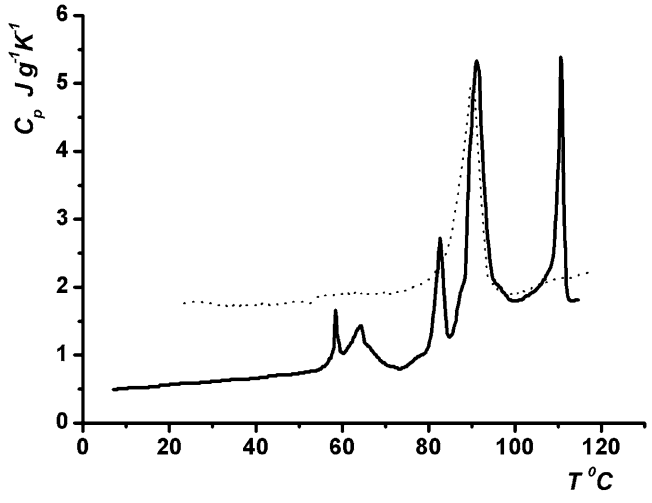

Fig. 6 **a** Dependence of the specific heat capacity of IRA phage on temperature in the case of low ionic strength of the solvent. **a′** Dependence of the specific heat capacity of IRA phage on temperature at reheating

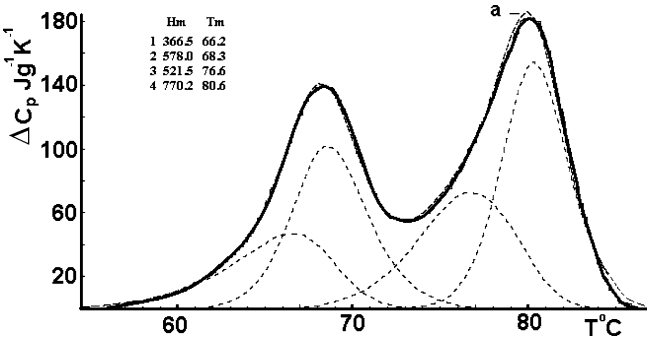

Fig. 7 *Solid line* dependence of the excess heat capacity of *FhuA* on temperature. *Dotted line* deconvolution of recorded calorimetric curve of *FhuA*. *Curve a* theoretical curve—the sum of four peaks. *Solvent* 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.05, 0.03% LDAO

tions adhere to an "all or nothing" principle. The theoretical curve (Fig. 7a) is the sum of four peaks and each of them is calculated by Vant-Goff's equation. So, based on the above discussion, we suppose that FhuA consists of four domains. The DNA ejection from T5 phage induced by FhuA protein was studied by means of viscometry method. The dependence of specific viscosity of T5 phage–FhuA complex on time is given on Fig. 8 ("square" line). The experiment was carried out at constant temperature of 25°C. During first 20 min the specific viscosity of the phage suspension is constant. Then FhuA is added, in a ratio of protein to phage of 900 to 1, resulting in a viscosity increases that appears to asymptotically approached a maximum. The specific viscosity value corresponding to this maximum (a.k.a., saturation) indicates complete DNA release from the head of phage. We experimentally determined that the minimum protein-to-phage ratio necessary for complete DNA release is 300 to 1 (Fig. 9).

The activity of FhuA to IRA phage was also studied. It should be noted that the host cell for IRA phage is *Salmonella typhimurium*, but it turned out that IRA phage also lyses *E. coli* K12 HO830 cells that over express FhuA, the *E. coli* strain that also serves as a host for T5 phage. Based on this apparent requirement for FhuA by IRA phage for adsorption to *E. coli* K12, we infer that FhuA probably serves as the host receptor for IRA phage. Given the morphological similarities between T5 and IRA phages, we have repeated the viscometric experiment shown from T5 in Fig. 8 ("star" line) but using IRA phage instead. Our experimental result, however, indicated no activity of FhuA to IRA, even in the case of very high concentration of the protein in phage suspension (FhuA/IRA ratio was 1,000/1, correspondingly). Hence other component of *E. coli* surface serves as receptor for IRA phage. To confirm this result it was decided to grow T5 phage on the *S. typhimurium* strain that serves as our host for phage IRA. We find, however, that T5 phage does not grow on this strain. Hence, this *S. typhimurium* strain does not appear to serve as a host cell for T5 phage. This may conclude that

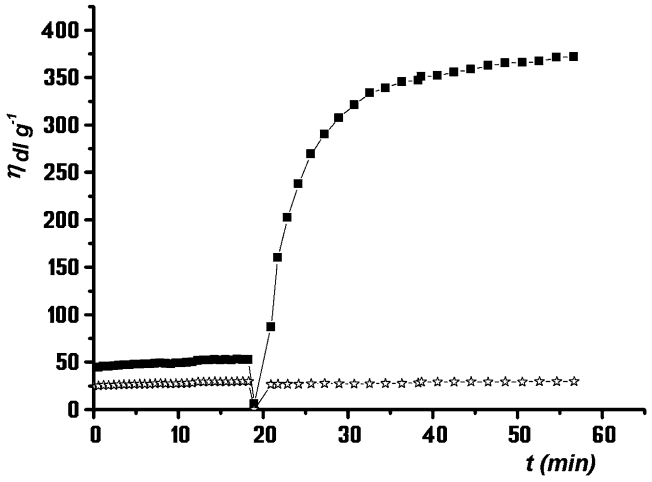

Fig. 8 *Square line* dependence of the specific viscosity of T5 phage suspension on time. T5 phage DNA ejection induced by *FhuA* receptor. The *FhuA*/*T5* ratio in the experiment is 900/1, correspondingly. *Star line* dependence of the specific viscosity of *IRA* phage—*FhuA* complex on time. The *FhuA*/*IRA* ratio in the experiment is 1,000/1, correspondingly. The FhuA was added in 20 min after starting of the experiment

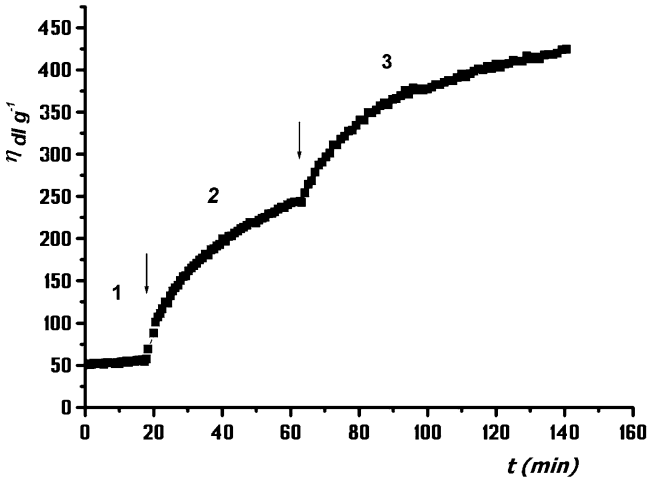

Fig. 9 Interval 1 dependence of the specific viscosity of T5 phage suspension on time. Interval 2 T5 phage DNA ejection induced by *FhuA* receptor (the *FhuA*/*T5* ratio in the experiment is 200/1, correspondingly). Interval 3 the *FhuA*/*T5* ratio in the experiment is 300/1, correspondingly

probably the outer membrane of above-mentioned bacteria does not contain FhuA.

Discussion

The viscometric results for IRA phage (Fig. 1) indicate that increasing solvent ionic strength leads to a change in the specific viscosity maximal value, but not to a change in the initial temperature of DNA ejection. This result suggests that the degree of DNA release from IRA phage depends on the ionic strength of the solvent, i.e., the higher is the ionic strength of the solvent the more phage DNA that releases into the solvent.

Considering that IRA and T5 phages are very similar morphologically and, moreover, that they can grow on the same bacterial strain (*E. coli* K12 HO830) we would suspect that their physico-chemical and biological properties would also be similar. However, the results of calorimetric and viscometric experiments have shown that biophysical properties of these phages are qualitatively distinct (Fig. 2). For example, the DNA ejection in the case of physiological conditions for T5 phage starts at a lower temperature and then occurs in two stages. The two stages of DNA ejection can be explained by the existence of a force associated with the T5 capsid, such as inside the head, which holds back the DNA from completing ejection. Consequently, only some part of the DNA is released at lower temperatures while complete DNA release occurs at higher temperature (Fig. 2b). It should be mentioned that one-stage DNA release from T5 phage is also observed, but occurs at a lower solvent ionic strength (Fig. 2a). This discrepancy in the temperature dependent of DNA ejection, which is solvent ionic-strength dependent, points to the opening of the T5 phage tail channel depending on electrostatic forces.

Also we have studied the ability of phage DNA to renaturate its structure by means of viscometry. It has been shown that for both phages DNA does not renaturate completely (Figs. 1, 2). It should be mentioned that even single breaks in the structure of renaturated phage DNA molecule are fixed by viscometer.

Not surprisingly, given the heterogenity of phage virions (which consist of DNA along with numerous proteins each displaying different structural organizations), calorimetric investigation of bacteriophages under thermal denaturation produces complicated heat absorption curves. Though these curves are complicated, it nevertheless is a possible to determine during what temperature interval a given phage component (DNA, phage head, tail) denatures. This is done by using the tendency of dsDNA molecules to renaturate given appropriate (e.g. cooler) physiological conditions. The above-mentioned property of DNA allows a precise determination of the temperature interval at which phage DNA melts that is based on a repeat determination of the calorimetric curve. Correspondingly, those peaks that do not reappear during a repeat determination of the microcalorimetric curve represent a denaturation of phage proteins, since these phage particle consist of only DNA and protein.

By microcalorimetric study we show that for both phages no one capsid protein is renaturated upon cooling, but instead that only the phage DNA possesses renaturation ability. Moreover, the microcalorimetry method indicates that complete phage DNA renaturation is achieved. It should be mentioned that multiple repeat of cyclic denaturation (heating–cooling–heating) always leads to appearance of phage DNA heat absorbance peak, with neither the phage DNA denaturation

temperature nor the enthalpy at that does not change. Analysis of viscometric curves also gives information regarding the temperature region in which phage DNA melts. There are capsid proteins in IRA phage's structure which denature both at low temperature, i.e. before phage DNA denaturation, and after DNA denaturation (Fig. 4). Viscometric investigation of IRA phage allowed further characterization of each temperature interval, in which heat absorption peak was observed to be the peak of definite component of phage particle. Hence, based on these viscometric results we infer that the intensity of DNA ejection from phage IRA is maximal at 73°C and, furthermore, that IRA phage DNA denatures at approximately 92°C (Fig. 1). These values suggest that the heat absorption peaks observed at 55–70°C (Fig. 4a) cannot be responsible for phage genome ejection, since ejection has a heat absorption peak of 73°C. Moreover, the observed heat absorption in the temperature region of 55–70°C cannot be significant head-protein denaturation because a thermally disrupted phage head would lead to changes in phage DNA structure and possibly a loss of normal DNA ejection ability. Furthermore, dramatic change in DNA structure as would occur given significant DNA leakage through holes in the phage head, which could be produced by head-protein denaturation, should be observable via viscometric means, but were not.

IRA phage head proteins most likely melt within the temperature area of 75–85°C, because the high temperature cooperative heat absorption peak ($T_{max} = 110$°C) can probably be related to the denaturation of the phage tail. The IRA phage tail can be characterized in terms of homogeneity of proteins resulting in a denaturation that occurs over a very narrow heat absorption peak, with half-width in an order of 1°C (Fig. 4). We additionally suppose that denaturation of the phage tail occurs at higher temperatures because of the existence of strong inter-protein interactions over the long noncontractile proteins making up the IRA phage tail.

We additionally investigated the influence of ionic strength on thermodynamical properties of phages T5 and IRA. We found that solvent at low ionic strength does not allow T5 phage DNA to renature (Fig. 5). This result compares with high ionic strength, where DNA completely renatures (Fig. 4). It is easy to observe from the Fig. 6 the decrease of the dependence of specific heat capacity on temperature, which indicates on the aggregation passes after the DNA denaturation. We suppose that DNA does not renature at low ionic strength in these experiments as a consequence of interference by capsid protein aggregations (Fig. 6). Consistently, we have shown (Ivanova et al. 2003; Mdzinarashvili et al. 2004; Mrevlishvili et al. 1990, 1992, 1999; Khvedelidze et al. 2004) for a number of phages with icosahedral head symmetry that DNA ejection from phage capsid is not accompanied by either endo-, or exo-heat effects.

For the biophysical investigation of features of the functional 3D structure of membrane proteins, the polar and nonpolar molecules should be simultaneously present in investigated solvent. These properties are achieved by employing detergents. Addition of detergents to an aqueous solution allows membrane proteins to form ordered structures both in their polar part (aqueous environment) and in their nonpolar part, which contacts with nonpolar area of detergent molecule. We suppose that conformation of the protein, dissolved in detergent-aqueous solvent can form ordered structures that are analogous to the in vivo protein structure within membranes. In the case of absence of detergents, by contrast, proteins can form aggregates, becoming inactive.

Two transitions of the calorimetric curve found in Fig. 7 allow us to conclude that the denaturation process of FhuA is not a transition between two stable states, but instead that there is one more intermediate thermodynamic stable state. That is, as temperature increases the membrane protein passes from the native state through a number of intermediate macro-states until finally the denaturated state is reached. Thus, this process does not obey the principle of ''all or nothing'' (native vs. denatured) but instead FhuA has intermediate stable states. On the other hand, the existence of two peaks does not mean that there are only two domains in the structure of FhuA. Considering such evident characteristics of an excess heat absorption peak, as its altitude, half-width, and square, the deconvolution method can be used, which allows one to obtain all the necessary information regarding the number of domains found within a protein.

The deconvolution of experimental curve is given on Fig. 7. The theoretical absorption peaks correspond to the domains, by which the structures composing the native molecule are shown as dotted lines. Each peak is elementary, considering that these transitions obey to ''all or nothing'' principle. The theoretical curve (dotted line) is the sum of four peaks and each of them is calculated by Vant-Goff's equation. So, based on the above discussed we suppose that in a chosen conditions FhuA consists of four domains. Considering the fact that one domain on average consists from 100 to 200 amino acid residues (Alberts et al. 1986; Voet et al. 2000) and in the case of FhuA, which has 714 amino acid residues in total and its high-ordered structure (Boulanger et al. 1996; Bonhivers et al. 2001), it is not surprising that it can consists of four domains.

Besides, we hardly can imagine that FhuA's two-domain structure is enough to conduct ferrichrome transfer through the membrane, FhuA's function in *E. coli* metabolism. From our point of view this translocation must occur in a more complicated manner, with ferrichrome transfer taking place at the expense of conformational changes in FhuA. A two-domain FhuA structure is too simple to realize this function. Hence, we believe that the existing model of FhuA function in membrane should be re-evaluated.

Finally, from our experimental results we conclude that the start of the DNA ejection process from the phage particle occurs without additional energy from either a physical (for example temperature) or chemical

(for example ATP molecules) source. The energy that is necessary for the transfer of the genetic material from phage capsid to host cytoplasm is imbued into the phage particle during the assembly process in the host cell. This spare energy of the phage is part of the structural organization of the phage genome inside the phage head.

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