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Characterization of the Cholesteric Phase of Filamentous Bacteriophage *fd* for Molecular Alignment

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Residual dipolar couplings arise from small degrees of alignment of molecules in a magnetic field and have proven to provide valuable structural information. Colloidal suspensions of rod-shaped viruses and bacteriophages constitute a frequently employed medium for imparting such alignment onto biomolecules. The stability and behavior of the liquid crystalline phases with respect to solution conditions such as pH, ionic strength, and temperature vary, and characterization should benefit practical applications as well as theoretical understanding. In this Communication we describe the pH dependence of the cholesteric liquid crystalline phase of the filamentous bacteriophage *fd* and demonstrate that the alignment tensor of the solute protein is modulated by pH. We also report the interesting observation that the relative sign of the residual dipolar coupling changes at low pH values. In addition, we demonstrate that the degree of alignment inversely scales with the lengths of the phage particles for phages with identical mass and charge per unit length.

Key Words: **residual dipolar couplings; bacteriophages, *fd*, Pf1, liquid crystal.**

INTRODUCTION

Recently, NMR methods have been developed which focus on the extraction of structural restraints characterizing long-range order. The major breakthrough with respect to any potential routine use of dipolar coupling derived structural restraints was the demonstration that tunable degrees of molecular alignment can be achieved by adding aqueous liquid crystalline media to the molecule under investigation (*1*), and a variety of different media have been developed for this purpose (for reviews see *2, 3* and references cited therein). One of the media that is becoming more and more popular consists of a suspension of filamentous phage, either *fd* (*4*) or Pf1 (*5*). Suspensions of charged, rod-shaped viruses, such as tobacco mosaic virus and filamentous bacteriophages *fd*/M13 and Pf1, are known to undergo a magnetic field induced isotropic–nematic phase transition at moderate concentrations (*6–8*). We investigated and developed methodology based on the *Escherichia coli* bacteriophage *fd*. The phage particles are negatively charged rods of 6.6-nm diameter and 880-nm length, in which a cylinder of coat proteins is arranged in a helical fashion around a single-stranded DNA genome. Concentrations commonly used for alignment purposes are 20–30 mg/ml and the magnitude of the alignment is influenced by the concentration of the liquid crystalline phase. The large viral particle size leads to high macroscopic viscosity; however, the microscopic tumbling rates of the dissolved macromolecules are not affected.

Figure 1A displays the correlation between the measured residual $^1D_{NH}$ dipolar couplings for the B1 domain of protein G in colloidal suspensions of *fd* for different pH values, but identical ionic strengths and protein and phage concentrations. The corresponding parameters calculated for the alignment tensor as well as rms and the correlation coefficient for observed versus calculated residual dipolar couplings are listed in Table 1. As can be easily appreciated, the orientation of the tensor changes with pH. In particular, the rhombicity decreases with decreasing pH and approaches a low value of 0.31, comparable to that of 0.23 previously observed for alignment in lipid bicelles. For the data presented in Fig. 1A all $^1D_{NH}$ values were calculated by subtracting the splitting measured in the anisotropic phase from that in the isotropic one. This takes into account that the sign of the direct $^1J_{NH}$ coupling is negative. It is immediately apparent from the graphs displayed in Fig. 1A that the sign of $^1D_{NH}$ at low pH (pH 3.0) is inverted compared to the high pH values. A similar anti-correlation between residual dipolar couplings was previously observed for dipolar couplings measured in the surfactant liquid crystalline phase CPBr/hexanol/NaBr at pH 3 versus those in phage at pH 5 (*9*). Figure 1B shows the correlation between $^1D_{NH}$ dipolar couplings measured at the identical low pH (pH 3) in 5% liquid crystalline medium of CPBr/hexanol in 10 mM glycine–HBr buffer containing 25 mM NaBr and in ~30 mg/ml *fd* in 10 mM citrate buffer at 25°C. It is obvious that the sign of the measured $^1D_{NH}$ dipolar couplings is identical for equivalent residues in both media, demonstrating that the change of sign is related to the low pH. For the same pH, the surface charge distribution on the GB1 domain will be identical with an overall positive net charge, and the anisotropic

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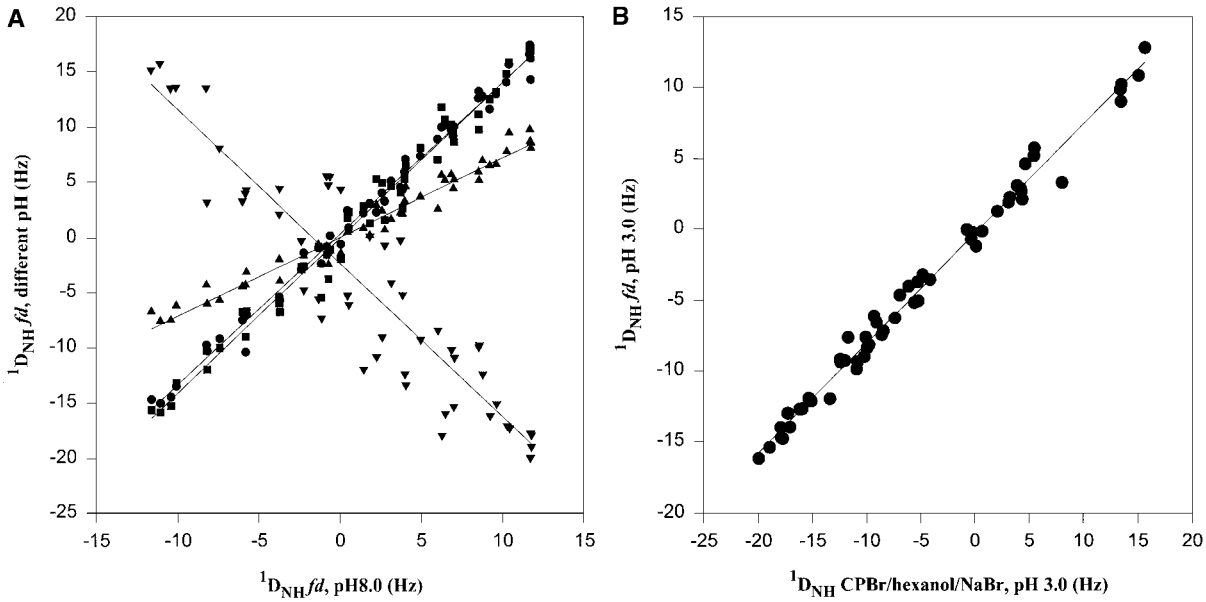

FIG. 1. (A) Correlation between the measured residual ^1H–^{15}N dipolar couplings for GB1 in the nematic phase of *fd* (~30 mg/ml) at different pH values. (●) pH 6.6, 10 mM sodium phosphate buffer; (■) pH 6.0, 10 mM sodium phosphate buffer; (▲) pH 5.0, 10 mM sodium phosphate buffer; (▼) pH 3.0, 10 mM sodium citrate buffer. The pH 8.0 sample contained 10 mM Tris–HCl buffer. Samples were prepared in the following manner: The pH 8.0, 6.6, and 6.0 samples were prepared by mixing phage and GB1 in the respective buffers. For the pH 5.0 and 3.0 samples, a stock solution of *fd* was diluted to ~0.3 mg/ml and dialyzed exhaustively against 10 mM sodium phosphate buffer or 10 mM sodium citrate buffer, respectively. The solutions were concentrated using a centricon filtration unit (10 kDa cutoff). GB1 solution was prepared by dialysis against the respective buffer and the appropriate amount of protein solution was added to the phage solution. Liquid crystal formation was assessed by observing optical birefringence through crossed polarizers and by measuring the splitting of the low field tryptophan NεH resonance in a 1D spectrum of ^{15}N-labeled GB1. (B) Correlation between experimental values of $^1D_{NH}$ measured in the nematic phase of *fd* (~30 mg/ml; in 10 mM sodium citrate buffer, pH 3.0, 90% H_2O/10% D_2O) versus those obtained in the liquid crystalline phase formed by 5% CPBr/hexanol ((1/1.33; w/w) in 10 mM glycine–HBr buffer, pH 3.0, 10 mM NaBr, 90% H_2O/10% D_2O). The correlation coefficient is 0.994. The ^1H–^{15}N splittings ($^1J_{NH} + {}^1D_{NH}$) were measured from ^{15}N ^1H HSQC spectra with no decoupling in the ^{15}N dimension on a Bruker DMX 500-MHz spectrometer at 25°C. The error in the measured $^1D_{NH}$ values ranges from 0.2 to 0.4 Hz.

medium carries a positive charge as well; the alignment should be dominated by repulsive forces.

It was shown previously that the principal axes of the alignment tensor coincide with the solute molecule's rotational diffusion tensor if the alignment is purely steric and dominated by the shape of the solute molecule (*10*). This was the case for ubiquitin in neutral phospholipid bicelles. This need not necessarily be the case for other proteins and media, and in

TABLE 1
Alignment Tensor for GB1 and rms between Observed and Calculated Residual Dipolar Couplings, $^1D_{NH}$, in Different Liquid Crystalline Media[a]

Liquid crystal	α (degree)	β (degree)	γ (degree)	D_A (Hz)	R^b	rms (Hz)[c]	r^d	Number of D_{NH}
fd pH 8.0 (30 mg/ml)[e]	8.3	102.4	66.1	6.09 ± 0.02	0.606 ± 0.007	1.27	0.9807	50
fd pH 6.6 (29 mg/ml)	7.1	100.8	65.9	8.55 ± 0.01	0.616 ± 0.003	1.56	0.9866	50
fd pH 6.0 (29 mg/ml)	12.1	99.4	66.0	8.94 ± 0.01	0.623 ± 0.001	1.57	0.9852	50
fd pH 5.0 (30 mg/ml)	13.3	100.5	65.1	4.83 ± 0.01	0.488 ± 0.004	1.47	0.9637	55
fd pH 3.0 (32 mg/ml)	13.6	76.0	159.2	10.45 ± 0.01	0.408 ± 0.006	1.48	0.9889	47
CPBr/hexanol/NaBr pH 3.0 (5% w/v)	11.1	78.9	160.9	8.27 ± 0.01	0.315 ± 0.004	1.41	0.9854	51
bicelles pH 7.0 (5%)	43.2	55.7	235.8	9.63 ± 0.09	0.233 ± 0.002	1.50	0.9894	50

[a] Singular value decomposition was employed for best fitting the alignment tensor to the observed residual dipolar couplings (*21*). The Euler angles α, β, and γ specify the orientation of the alignment tensor **D** relative to the coordinate frame of the 1.1-Å X-ray structure.

[b] R, Rhombicity defined by D_R/D_A, with D_A and D_R representing the axial and rhombic components of the tensor **D**, respectively.

[c] rms difference between the observed and calculated values of $^1D_{NH}$ based on the known orientations of the NH vectors in the X-ray structure.

[d] Linear correlation coefficient between predicted and observed residual dipolar couplings.

[e] Concentration is accurate to ±1 mg/ml.

particular liquid crystalline phases composed of charged particles frequently exhibit considerable contributions from electrostatic interactions in their alignment properties such that the alignment tensor cannot be predicted from the shape of the solute (*11*). At pH values above its isoelectric point of pH 4.2 *fd* phages are negatively charged and at pH 7 in water a charge density of 10 e/nm has been reported. At the low pH value of 3, the phage particles will be positively charged. Adding salt to the phage liquid crystal phase screens the electric repulsion between the particles, thereby essentially decreasing the effective diameter of the phage to the bare diameter of 6.6 nm at high salt (>300 mM) concentrations (*12*). As a result, the coexistence concentration for isotropic and anisotropic boundary increases with increasing ionic strength (*12*). We therefore took great care to insure that identical buffer concentrations were used for all samples to avoid any possible influence of differences in the phase boundary between isotropic and liquid crystal phases. Since the p*I* for GB1 is 4.5, the protein will also be positively charged at pH 3 and both molecules should repel each other. From the trend observed for the value of the rhombic component of the alignment tensor, it appears that high rhombicity is indicative of a significant charge component in the interaction for the same shape solute. It therefore may be appropriate to consider the shape of the solute in terms of an effective shape, which would take the charge distribution on the surface into account, rather than the molecular shape for the prediction of alignment tensors. It should be noted that no significant conformational changes occur for GB1 even at pH 3. The protein exhibits extreme stability with a urea unfolding transition midpoint of 3.7 M at pH 2 (*13*); thus the change in alignment tensor cannot be attributed to a change in protein structure. Whether any quantitative conclusions can be drawn from the present data set will have to await further investigation.

Figure 2 shows a plot of the measured $^1D_{NH}$ dipolar couplings versus residue number for the GB1 domain in colloid suspensions of three different phages for identical solute concentration, pH, and ionic strength conditions. The values obtained from the *fd* liquid crystalline phase at a concentration of 30 mg/ml are identical, within the error of the data, to those measured for Pf1 at 16 mg/ml. The third set of data was measured for the liquid crystal phase formed by a mutant *fd* phage. The characteristic property of this *fd*K48A phage is an increase in length over wild-type *fd* of ca. 35% (*14*). The *fd*K48A phage is mutated in the gene VIII protein of *fd*, resulting in a single amino acid change in the major coat protein. The coat proteins in the phage virions are arranged in a right-handed helical array with five subunits in the 16.26-Å axial repeat (*15*). The length of the packaged single-stranded DNA genome in the mutant phage is not changed compared to the wild-type; it simply is more stretched out inside a longer virion (*16*). Likewise, Pf1 closely resembles *fd* in morphology; it exhibits an identical particle diameter of about 6.6 nm, the mass per unit length is very similar (18,300 Da/nm for *fd* and 18,600 Da/nm for Pf1) and has a similar p*I* value (4.0 for *fd* and 4.2 for Pf1), and the charge density for both phages is identical over the pH 7–8 interval (*17*). The only gross morphological distinction between Pf1 and *fd* is their

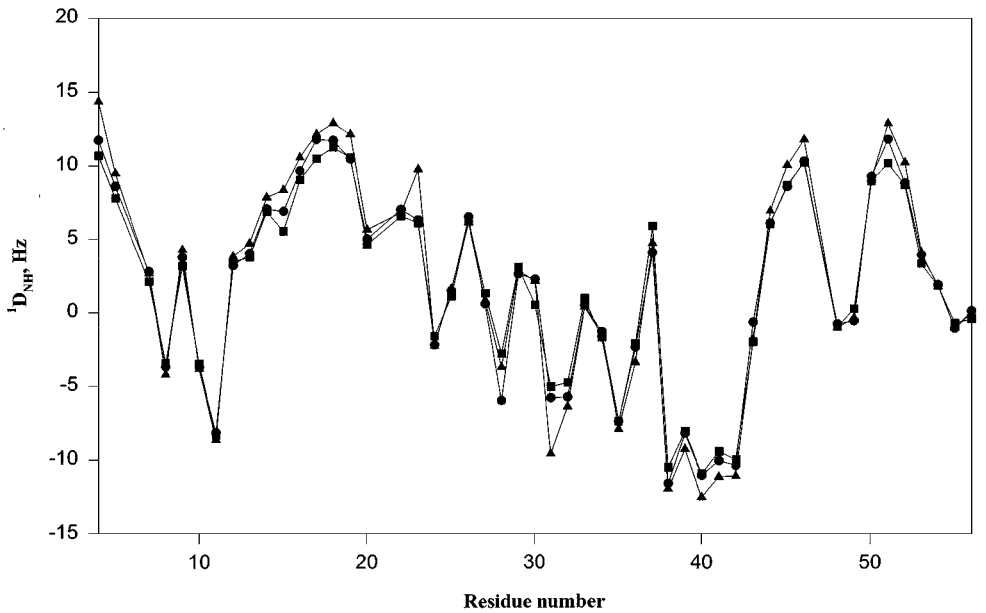

FIG. 2. Plot of $^1D_{NH}$ measured at 500 MHz versus residue number for the GB1 domain (0.5 mM) dissolved in liquid crystalline phases of three different bacteriophages. The mass concentration of the different colloidal suspensions is (●) 30 mg/ml *fd*, (■) 16 mg/ml Pf1, and (▲) 30 mg/ml *fd*K48A. The data measured for the *fd*K48A suspension were scaled by 0.74 to take into account the 35% increase in length. All solutions contained 10 mM Tris–HCl, pH 8.0, 90% H_2O/10% D_2O. All measurements were carried out at 25°C.

respective length; Pf1 is approximately twice as long as *fd*. Thus all three virions have very similar architectures and can be treated for theoretical purposes as long, thin, semiflexible charged rods of identical diameter, charge density, and mass per unit length, but of different overall lengths. Khokhlov and Semenov generalized Onsager's (*18*) statistical mechanical treatment for the isotropic to nematic phase transition of a suspension of charged rigid rods to include systems of semiflexible chains (*19*). For colloidal *fd* solutions it was demonstrated experimentally (*12*) that the coexistence concentration (ρ_x) or the concentration at which the isotropic phase becomes absolutely unstable (ρ^*) compares well with predictions based on the Khokhlov–Semenov theory. In order to qualitatively interpret the data presented in Fig. 2 we use Eq. [1] for deriving the coexistence mass concentration in milligrams per milliliter

$$\rho_x = Mc_x/L^2D_{\text{eff}}, \qquad [1]$$

with c_x the dimensionless number concentration, M the mass of the individual particle, L the contour length of the particle, and D_{eff} the charge-dependent effective diameter. Given that the mass of the individual particle (M) equals the mass per unit length (m) times the length of the particle (L), Eq. [1] can be rewritten as

$$\rho_x = mc_x/LD_{\text{eff}}. \qquad [2]$$

For the present case of *fd*, *fd*K48A, and Pf1 the mass per unit length, m, is nearly identical and the effective diameter should be identical under identical ionic strength and pH conditions. Therefore, the coexistence concentration in milligrams per milliliter should be inversely proportional to the length of the phage particle. The size of the dipolar coupling is proportional to the degree of alignment, thus the fact that for identical mass concentrations in milligrams per milliliter of *fd* and *fd*K48A larger residual dipolar couplings are observed for the longer phage indicates a $1/L$ dependence. Indeed, taking the data for all three phages, a surprisingly good $1/L$ dependence is observed. Nearly identical values are measured for the sample in *fd* and Pf1, with the latter at $^1/_2$ the phage concentration (mg/ml), consistent with the fact that Pf1 is twice the length of *fd*. The measured $^1D_{NH}$ dipolar couplings for GB1 in *fd*K48A are a factor of 1.35 larger than those measured in *fd* at identical phage concentrations (mg/ml), corresponding to their length ratio L_{fd48K} (1200 nm)/L_{fd}(890 nm). Thus it is possible to predict and tune the degree of alignment using defined concentrations of various-length phage particles.

In conclusion, we have shown that the molecular alignment tensor of solute molecules aligned in liquid crystalline media of colloidal phage suspensions is modulated by pH. Both the axial component ($D_A{}^{NH}$) and the rhombicity of the molecular alignment tensor are affected. For the positively charged phage particles at low pH we observe the largest magnitude of the tensor, corresponding to a greater degree of alignment of the solute or greater order within the liquid crystal. This observation may be related to results obtained by X-ray diffraction of hydrated fibers which indicate that at pH 2 the individual virions in *fd* fibers are more highly ordered than at pH 8 (*20*). It should also be pointed out that, in contrast to numerous reports, it is possible to prepare liquid crystalline phases of phage at low pH (pH 3 and below), at least for *fd*, and that the orientation of the alignment tensor for GB1 in the low pH phage liquid crystal phase is very similar to the one previously determined for the low pH surfactant liquid crystal phase formed by cetylpyridinuim bromide/hexanol/NaBr (see Table 1). Finally, using different-length phages, we demonstrate that the size of the residual dipolar $^1D_{NH}$ coupling is related to the length of the phage as expected from Khoklov–Semenov–Onsager theory.

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