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X-ray-scattering of turkey skeletal-muscle troponin C in solution at low pH

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The solution structure of troponin C from turkey skeletal muscle was studied at low pH by small-angle X-ray-scattering. We find that troponin C at pH 5.3 in the presence of Mg^{2+} has a triaxial radius of gyration and maximum dimension comparable with those of the crystallized protein. However, the relative disposition of domains is more similar to that found for the highly homologous rabbit protein in solution at pH 7.4.

INTRODUCTION

The X-ray structure of the regulatory protein troponin C from turkey skeletal muscle has been solved to high resolution [1]. The conditions of crystallization, i.e. low pH (5.0) in the presence of Ca^{2+} ions, resulted in only the two high-affinity metal-ion-binding sites of the *C*-terminal domain being occupied. The two low-affinity sites of the *N*-terminal domain remained free of metal ions. This situation has been shown to exist also in the case of Ca^{2+} binding to the highly homologous rabbit protein if the pH is kept low [2]. The unusual nature of the crystal structure, namely a dumbbell-shaped molecule with two globular ends, one containing the *C*-terminal domain and the second the *N*-terminal domain, encouraged investigations of whether such a conformation is preserved under physiological conditions, and how it depends on the number of metal ions bound. In each of the X-ray-scattering studies published to date, one concerned with Mg^{2+} binding to rabbit skeletal-muscle troponin C at neutral pH [3] and the second with no, two or four Ca^{2+} ions binding at neutral pH [4], the conclusions were similar. The separation between the two globular ends of the structure in solution is either smaller or less distinct than in the crystal lattice. Wang *et al.* [5] have measured resonance-energy transfer in rabbit skeletal-muscle troponin C in solution and found that the molecule appears to undergo a conformational transition. The distance between the donor in the *N*-terminal domain and the acceptor attached at Cys-98 in the *C*-terminal domain increases from 27 Å (i.e. 2.7 nm) to greater than 52 Å (i.e. 5.2 nm) as the pH is lowered from 6.8 to 5.0. In the present paper we give solution scattering data measured at low pH that are relevant both to the structure under crystallization conditions and to the measurement reported by Wang *et al.* [5].

MATERIALS AND METHODS

Sample preparation

Turkey skeletal-muscle troponin C was obtained as a pure powder from Dr. O. Herzberg (CARB, Gaithersburg, MD, U.S.A.). Sodium phosphate buffer, pH 5.3, was prepared to which 2 mM-$MgCl_2$ was added. The protein was dissolved to a concentration of 20 mg/ml with the final volume of the sample 150–200 μl. The sample was then dialysed against the same buffer (volume ratio 1:500) overnight at 4 °C with stirring. Because of the small volume of the sample, standard dialysis procedure could not be used. Rather, the sample was placed in an Eppendorf tube, in the cap of which a hole had been cut. A square piece of dialysis membrane (Spectrapor, 3500 M_r cut-off) was placed across the mouth of the tube and the cap was sealed shut upon it. The tube was then inverted, with care being taken that no air bubbles interposed themselves between the specimen and the membrane, and inserted into the beaker containing the dialysis buffer. In some cases, after dialysis the specimen was further concentrated in a vacuum concentrator (Micro Pro Di Con). Scattering specimens were prepared by dilution from the stock solution. Protein concentration was determined by u.v. spectroscopy by using the absorption coefficient 0.132 at 259 nm for a 1 mg/ml solution of turkey skeletal-muscle troponin C. All solutions were kept either frozen or at 4 °C until needed, in the latter case for not longer than 9 days. The dialysis buffer provided the appropriate reference for the scattering experiments.

Small-angle X-ray-scattering experiments

Solutions of troponin C (6–20 mg/ml) prepared as described above were placed in a 1.5 mm-diameter quartz X-ray capillary and inserted into a brass block through which a chilled (15 °C) water/ethanol mixture flowed. A Phillips sealed-tube X-ray generator was operated at approx. 40 kV and 34 mA. The line-profile Ni-filtered Cu X-radiation was collimated and further monochromated by a single Franks mirror and a series of slits and height-limiters. The distance between the sample and the detector was approx. 46 cm. The scattering profile was recorded by a linear-position-sensitive detector of the delay-line type [6] and stored in a Z-80-based microprocessor unit as a 256-channel histogram. At the completion of each experiment the data were sent via a hard-wire link to an IBM 3081 computer for further

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analysis. Duration of experiments ranged from 10.5 to 24 h.

Data analysis

For each specimen, two parallel experiments were run, namely protein and then buffer only, all other parameters remaining unchanged. The buffer scattering curve was subtracted from that of the protein solution after normalization with respect to the integrated intensity of the main beam. Data were desmeared by using the Lake procedure [7] and Guinier analysis [8] was performed to obtain the triaxial radius of gyration, R_G. Analysis of the background-subtracted smeared profile was also performed by using the indirect transformation procedure (ITP) of Glatter [9]. The unsmoothed data were deconvoluted from the beam shape, and the noise was treated as statistical in origin. The spline fit curve $I(q)$ was then used to calculate the distance distribution function:

$$P(r) = (1/2\pi) \int_0^\infty I(q) \cdot q^2 \cdot [\sin(qr)/qr] \cdot dq$$

where $q = (4\pi/\lambda) \cdot \sin\theta$, 2θ is the scattering angle and $\lambda = 1.54$ Å for Cu radiation. This expression gives the frequency of occurrence of vectors of length r, weighted by the electron density at either end of the vector. The radius of gyration is obtained from:

$$R_G^2 = \int_0^\infty P(r) r^2 \cdot dr / 2 \int_0^\infty P(r) \cdot dr$$

The infinite integrals were approximated by finite sums, restrictions on sample intervals and range being in accordance with information theory. Glatter [9] has shown that R_G calculated in this way is more reliable for many practical examples of non-isometric particles than the traditional Guinier method. The particle shape is reflected in the shape of $P(r)$, and the presence of interparticle interference is discernible.

Model calculations

Any model of arbitrary shape and internal structure can be approximated by a large number of very small spheres if they are smaller than the smallest structural details of interest. In order to simulate the low-angle solution scattering from the structure of troponin C as determined by X-ray crystallography the envelope of the protein was overlaid by a three-dimensional grid of points with grid spacing 2.5 Å. As in the previous studies [3,4], bound water molecules were not explicitly included. To each point was assigned a sphere, the radius of which was chosen so that the final volume approximated closely the molecular volume. The resulting overlaps do not affect the scattering curve to the resolution with which we work [10]. The scattering intensity of the aggregate of $n = 823$ spheres can then be computed by the Debye equation [11]:

$$I(q) = \sum_{i=1}^{n} \phi_i^2(q) + 2 \sum_{i=1}^{n-1} \sum_{k=i+1}^{n} \phi_i(q)\phi_k(q) \cdot [\sin(qr_{ik})/qr_{ik}]$$

where $\phi_i(q)$ is the scattering factor of the ith sphere and r_{ik} is the distance between centres of the ith and kth spheres. The resulting profile is essentially the shape factor of the molecule at infinite contrast. Considering that the data used for comparison did not exceed a resolution of about 20 Å, this procedure, which neglects intramolecular density fluctuations, is suitable. The scattering profile was then treated in an analogous way to the experimental data and the ITP procedure was performed. The resulting $P(r)$ functions could be directly compared.

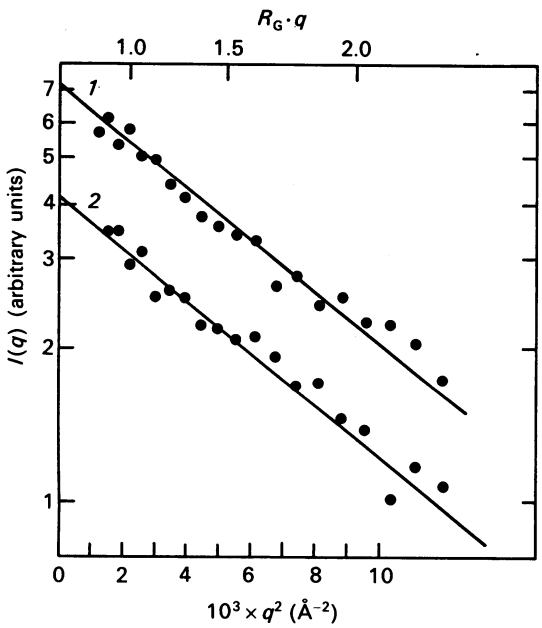

Fig. 1. Guinier plots for turkey skeletal-muscle troponin C

Semi-logarithmic plots of scattering intensity $I(q)$ versus q^2 for turkey skeletal-muscle troponin C in sodium phosphate buffer, pH 5.3, with addition of 2 mM-$MgCl_2$ at protein concentrations (curve *1*) 19 mg/ml (1020MGC) and (curve *2*) 12.2 mg/ml (1023MGA). Data were measured as described in the Materials and methods section. The region in which linear-regression analysis was performed extended to $R_G \cdot q = 1.6$.

RESULTS AND DISCUSSION

X-ray-scattering experiments on turkey skeletal-muscle troponin C were performed as described above. Characteristic low-angle profiles for two different concentrations are shown in Fig. 1. A semi-logarithmic plot of $I(q)$ versus q^2 shows that there is a linear dependence in the range between 1.2×10^{-3} Å^{-2} and 5.9×10^{-3} Å^{-2}. Linear-regression analysis in this region and the Guinier approximation $R_G \sim (-3\{d[\ln I(q)]/d(q^2)\})^{\frac{1}{2}}$ gives $R_G = 20.5$ Å for curve *1* and $R_G = 21.0$ Å for curve *2*. The fact that the two data sets do not show a significant change in slope suggests that at these concentrations little intermolecular interference remains. The values of the radius of gyration are intermediate between those measured by Hubbard *et al.* [4] (~ 20 Å) and Heidorn & Trewhella [3] (21.7–22.4 Å) at comparable concentrations. No sharp change of slope at low angles, which can indicate the presence of large aggregates, was observed.

A plot of R_G^2 versus concentration for several different specimens is shown in Fig. 2. There is, in this low concentration region, very little dependence of R_G on concentration. This is interpreted to mean that there are minimal residual intermolecular interference effects in this range [12]. In the absence of data at high protein

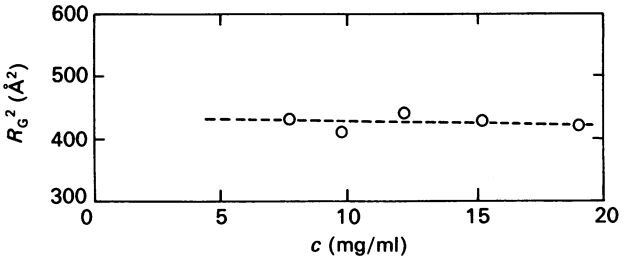

Fig. 2. Concentration-dependence of R_G for turkey skeletal-muscle troponin C

The values of the radius of gyration were calculated from the angular dependence of X-ray-scattering by using the Guinier approximation. R_G^2 is plotted as a function of protein concentration, any non-zero slope being indicative of interparticle interference.

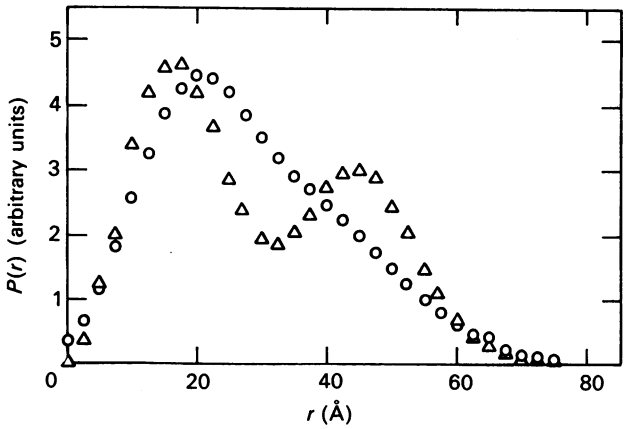

Fig. 3. Distance-distribution functions $P(r)$ for turkey skeletal-muscle troponin C

The frequency of occurrence of vectors of a given length within the troponin C molecule was derived by the indirect transformation procedure of O. Glatter from (△) the scattering profile computed from the crystal structure as derived in the text and (○) the experimental data set (c = 19 mg/ml).

concentration, we were unable to perform a reliable extrapolation to infinite dilution.

In Fig. 3 we have plotted the distance distribution function $P(r)$ for both the data set at 19 mg/ml and that calculated from the crystal structure as described above. The scattering between 0.03 Å^{-1} and 0.39 Å^{-1} was fit with 25 spline functions. $r_{max.}$ was chosen as 85 Å on the basis of the length of the crystal structure (75 Å) and was found to be satisfactory for the experimental data set as well. R_G calculated for the crystal structure is 23.0 Å and for the structure in solution is 22.7 Å, similar to the values found earlier [3,4]. Differences observed here between the R_G determined by the Guinier method and that determined by indirect transformation were also consistently found by Hubbard *et al.* [4]. For both model and data, the frequency of occurrence of vectors is maximal near 20 Å, and there is a long tail indicative of a prolate shape with maximum vector between 70 and 75 Å. Error bars indicate the uncertainty due to the propagation of the statistical fluctuations in the raw data. $P(0)$ should be identically zero for both curves, and the extent to which this is not so indicates the error in subtracting the background. The most striking feature of the crystal structure, i.e. the secondary peak at 45 Å, is not present in the solution data. Rather there is a small shoulder at 40 Å. The trough between the two peaks has been filled in, indicating a greater occurrence of vectors smaller than 40 Å. A similar profile has been deduced from the measurements made on the Mg^{2+}-bound rabbit protein at neutral pH [3]; the profile in the presence of Ca^{2+} is less similar [4]. It has been suggested that the absence of the secondary peak may be due to a contraction or bending of the molecule in solution at neutral pH [3,4], thereby bringing the centres of mass of the globular domains into closer proximity. It was found [3] that, if a bend is placed in the interconnecting α-helix of the crystallographic model such that the segment of the helix associated with the *N*-terminal domain and the segment associated with the *C*-terminal domain make an angle of 65.4°, then agreement with the solution scattering data is significantly improved. However, the limitations of low-angle X-ray-scattering do not permit one to discount the existence of other satisfactory models. The possible effects of including bound water have also been investigated, but with rather contradictory results. The addition of a uniform hydration layer 1–4 Å thick did not significantly change the model distance-distribution function [3]. However, 180 water molecules randomly distributed within the protein's first hydration shell and with an electron density 22% greater than that of bulk water did bring the model scattering curve into somewhat better agreement with the experimental curve [4].

We conclude from the indirect transformation analysis presented here that turkey skeletal-muscle troponin C in solution at low pH in the presence of Mg^{2+} is an elongated molecule with maximum dimension and radius of gyration similar to that of the crystal structure, and with a deposition of domains similar to that found in solution at neutral pH. It is possible that the increase in donor-to-acceptor distance observed with decreasing pH by Wang *et al.* [5] may be due to some combination of local main-chain and side-chain re-arrangements. Hubbard *et al.* [4] have suggested that a polymorphism within the *N*-terminal domain may occur that could change specific atom-pair distances but that would leave the overall length of the molecule unchanged. Perhaps such behaviour is occurring here.

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