ACTIVATION VOLUMES FOR THE ROTATIONAL MOTION OF INTERIOR AROMATIC RINGS IN GLOBULAR PROTEINS DETERMINED BY HIGH RESOLUTION ^{1}H NMR AT VARIABLE PRESSURE

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1. Introduction

Protein dynamics has evoked considerable interest recently [1,2]. It has been shown by ^{1}H nuclear magnetic resonance (NMR), for example, that aromatic side chains of amino acid residues buried in the protein interior undergo rapid rotational motion [3–6]. This phenomenon indicates that the protein structure undergoes spatial fluctuations of sufficient magnitude to provide the necessary space for a ring to flip. From quantitative measurements of flip rates of the rings energy barriers ($\Delta G^{\ddagger}$), were determined which the protein has to overcome during the process of a ring flip [5]. To better understand this free energy barrier ($\Delta G^{\ddagger}$), the temperature dependence of the flip rates was measured and the free energy term was split into enthalpic and entropic contributions [5]. Here, we further characterize this dynamic process by a measurement of the volume work expended by the protein during the process of the ring flip. This contribution to $\Delta G^{\ddagger}$ was obtained from variation of the hydrostatic pressure yielding the activation volume ($\Delta V^{\ddagger}$).

The activation volume is easy to visualize as a transient volume change of the whole protein during the process of the ring flip. We have used the basic pancreatic trypsin inhibitor (BPTI) since in this protein all 4 tyrosines and 4 phenylalanines are assigned [4,6–8] and since for two of these rings the dynamic process of the ring flips is readily analyzed from the NMR spectra [1]. In addition a very accurate crystal structure of BPTI is available for the interpretation of the data [9].

Abbreviations: NMR, nuclear magnetic resonance; BPTI, basic pancreatic trypsin inhibitor

2. Materials and methods

The basic pancreatic trypsin inhibitor (Trasylol®, Bayer Leverkusen) was obtained from the Farbenfabriken Bayer AG. For the NMR studies the protein was dissolved in ^2H_2O and adjusted to p^{2}H 5.2. Afterwards the protein solution was heated to 85°C for 10 min to exchange all labile protons against deuterium. After lyophilisation the protein was dissolved again in ^2H_2O to obtain a 10 mM solution.

The ^{1}H NMR spectra were measured on a Bruker HX-360 instrument. The home-built high pressure apparatus used is analogous to the design in [10,11]. Fig.1 shows a schematic representation of the high pressure high resolution NMR cell. The sample tube is a thick walled pyrex glass capillary (o.d. 8 mm, i.d. 1 mm) which is sealed at the bottom. At the upper end the tube is extended to a long thin tail which is glued into the copper–beryllium cone of the autoclave. At the upper end the capillary is prolongated by a flexible teflon hose. A glass stopper at the top of the hose separates the protein solution from the surrounding oil. Pressures up to 1200 bar were generated by a standard hand pump and supplied via the flexible walls of the teflon tube. To avoid damage in case of a burst the high pressure capillary was fitted into a 10 mm glass tube which could be inserted into the standard 10 mm probe of the Bruker HX-360 instrument. The length of the capillary and the protecting tube was adjusted to locate the autoclave above the magnet dewar (~81 cm).

The ^{1}H NMR spectra were obtained without sample spinning in the Fourier transform mode. A total of 4000 scans were accumulated for each spectrum while the spectrometer was locked to the deuterium resonance of the solvent, ^2H_2O. Chemical

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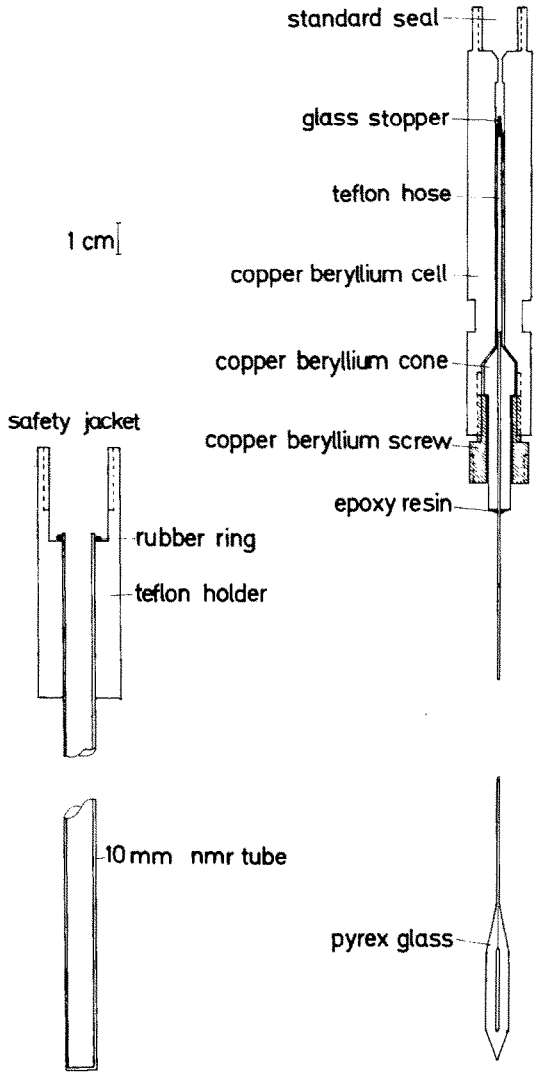

Fig.1. High-pressure NMR apparatus. The glass capillary is glued into a copper–beryllium cone which fits into the copper–beryllium autoclave. The end of the glass capillary is connected with a flexible teflon shrink hose. The protein solution contained in the teflon hose is separated from the pressurizing oil by a glass stopper. To prevent damage of the probe in case of a burst, the glass capillary is inserted into a long 10 mm NMR tube which is loosely connected with the autoclave by a teflon holder. The length of the capillary and the NMR tube was adjusted to keep the autoclave outside the magnet dewar.

shifts are quoted relative to the internal standard, sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate.

Flip rates were determined by computer simulation of the exchange broadened spectra. In this process a series of spectra was simulated with a stepwise increase of the flip rate. Since the rate was varied in small intervals a range of values for the rate was obtained which could fit the experimental spectrum. This range (typically 20%) is indicated as error bars in fig.3.

Activation volumes ($\Delta V^{\ddagger}$) were determined from the pressure dependence of the flip rates (k) according to the relation:

$$\left(\frac{\delta \ln k}{\delta p}\right)_T = -\frac{\Delta V^{\ddagger}}{k_B T} \quad (1)$$

3. Results

Fig.2 shows 360 MHz ^{1}H NMR spectra of the aromatic region of BPTI obtained with the non-spinning high-pressure sample cell. The resolution is comparable to that of spectra obtained from a normal sample spinning in a 5 mm tube. Due to the small sample volume and the poor filling factor ~4000 scans (~1 h measuring time) were necessary to obtain a reasonable signal-to-noise ratio.

With the experimental setup described pressures of up to 1200 bar could be applied. At 57°C no major spectral changes due to pressure occurred in this pressure range throughout the entire protein spectrum except for the dynamic effects described below. Thus the globular protein conformation remains essentially unchanged under the experimental conditions used. The effect of hydrostatic pressure on the flip rates of the aromatic rings is shown in fig.2. At 57°C and ambient pressure, Phe 45 has a symmetric AA'MXX' spectrum with the 2,6-protons at 7.38 ppm, the 4-proton at 7.64 ppm and the 3,5-protons at 7.86 ppm [5]. The resonances of the non-axial ring protons are slightly broadened because the flip rate is not sufficiently fast to cancel all exchange broadening [8]. This can be seen readily from the 3,5-proton resonance at 7.86 ppm which is well separated (closed arrow in fig.2). The flip rate of the side chain of Tyr 35 is much slower. Thus the spectrum of this aromatic ring is asymmetric, i.e., of the ABCX type with lines at 6.73, 6.80, 6.84 and 7.79 ppm. The one-proton lines are broadened, however, due to the slow flipping motion and are difficult to detect in the spectrum. In fig.2 the X-resonance at 7.79 ppm is labelled by an open arrow. With increasing pressure (fig.2) the XX'-resonance of the symmetric spin system of Phe 45 broadens while the X-resonance of

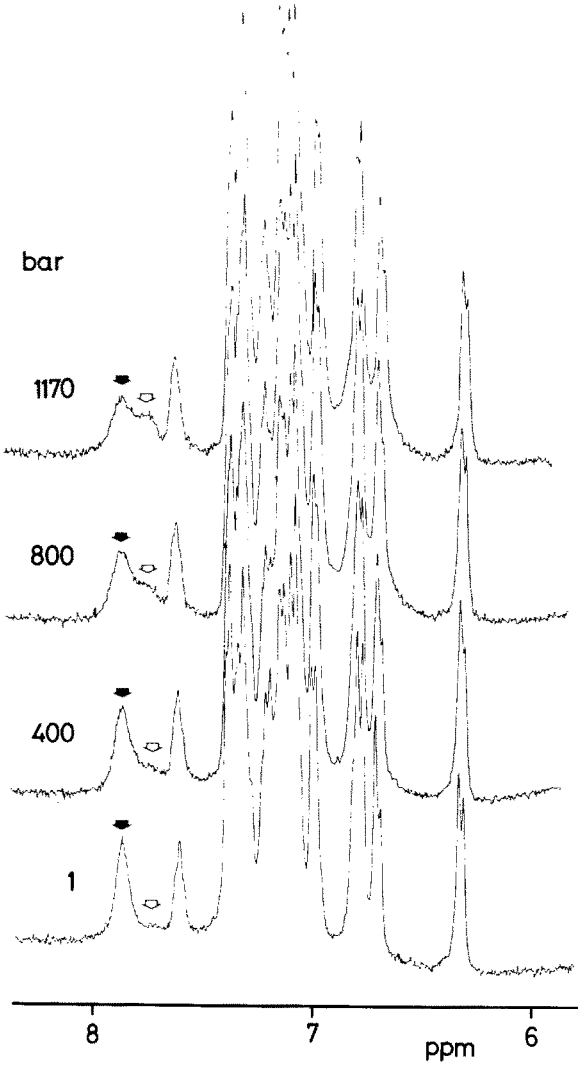

Fig.2. Aromatic region of the ^{1}H NMR spectrum of BPTI at 57°C at different hydrostatic pressures. The 3,5-proton resonance of Phe 45 and a one-proton resonance of Tyr 35 are labelled by a full and an open arrow, respectively [4,7]. The two-proton resonance of Phe 45 at 7.86 ppm broadens with increasing pressure indicating a decrease of the flip rate. The one-proton resonance of Tyr 35 at 7.86 ppm is very broad at 1 bar but sharpens with increasing pressure due to a decrease of the flip rate.

the asymmetric spin system of Tyr 35 sharpens, indicating that the rotation rates of both aromatic rings slow down with increasing pressure. This shows that the $\Delta V^{\ddagger}$ have positive signs, i.e., the activated states of the protein which allow the ring flips have a larger volume than the equilibrium state.

The flip rates of both aromatic rings were determined quantitatively by spectral simulation. In this simulation it was assumed that the 3- and 5-proton resonances of Phe 45 are separated by 235 Hz, a value determined at low temperature [3,5]. This assumption seems to be well justified since none of the immobilized rings showed sizeable temperature-dependent changes of the chemical shift. This is especially true for Tyr 35 where the well-resolved X-resonance at 7.79 ppm can be followed over 4–50°C [3,5]. Fig.3 shows a logarithmic plot of the flip rate of Phe 45 vs pressure. A similar plot was obtained for Tyr 35. The data were fitted according to eq. (1) and activation volumes of 50 ± 10 Å^3 and 60 ± 20 Å^3 were obtained for Phe 45 and Tyr 35, respectively. As fig.3 shows for Phe 45 no pressure dependence of $\Delta V^{\ddagger}$ was found within the experimental accuracy. Qualitative measurements were also carried out at other temperatures with similar results.

4. Discussion

The measurements of $\Delta V^{\ddagger}$ for the flips of internal aromatic rings in globular proteins exhibit new aspects for the understanding of protein dynamics. The

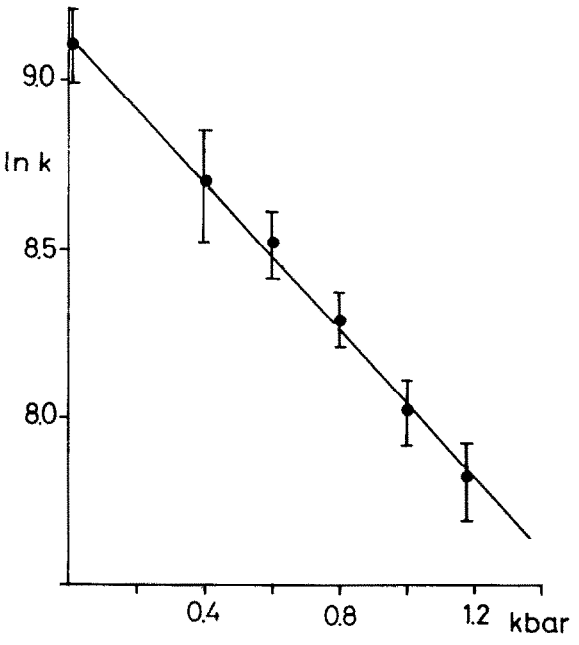

Fig.3. Logarithmic plot of the flip rate, k, (in s^{-1}) of Phe 45 vs pressure. Flip rates were determined by spectral simulation (see section 2).

observation of a sizeable $\Delta V^{\ddagger}$ indicates that the flipping of internal aromatic rings cannot be considered as a 'stirring motion' in a liquid-like medium, where close contact with the neighbouring groups would be preserved during the flipping motion. The measurements rather show that protein fluctuations occasionally provide some unoccupied space around the ring. Furthermore, the transient volume increase around an aromatic ring is not localized in a compressible ring environment but is transmitted to the protein surface since otherwise no $\Delta V^{\ddagger}$ would be observed. The $\Delta V^{\ddagger}$ determined experimentally from the flip rates of one aromatic ring is not buffered in a compressible ring volume expansion of the protein necessary for the flipping motion of the one individual ring considered. It cannot be proven for sure from the experiments described whether this volume expansion is restricted to the immediate environment of the ring, i.e., the creation of an unoccupied hole which enables the ring to flip, or whether the volume change is due to a global protein fluctuation which changes also the relative conformation and the volume of more remote regions of the protein molecule. From comparison of modified or homologous proteins we know that the protein fluctuations responsible for the flips of the aromatic rings are not global [1,8]. Thus we have tried to rationalize the volume fluctuations as local effects by comparing the size of the activation volumes measured with the dimensions of the aromatic side chain.

(1) The aromatic ring can be considered as an oblate rotational ellipsoid with half-axes of 3.5 and 1.7 Å [12]. The volume of this ellipsoid is 82 Å^3 and has to be compared with 164 Å^3 which is the volume of a sphere with the radius of 3.4 Å symbolizing the space occupied by the rotating ring. The difference of 82 Å^3 would correspond to a hypothetical activation volume and is somewhat larger than the experimental values determined. If we reduce the size of the sphere to an extreme short contact radius of 2.8 Å as suggested in a theoretical study [13] the hypothetical activation volume is only 46 Å^3 which is comparable to the experimental values.

(2) The activation volumes may be visualized on the basis of the X-ray conformation. Considering the crystal structures, proteins are rather densely packed. With the approximation that the single atoms of a protein can be represented as hard spheres with characteristic Van der Waal's radii there remain small void volumes throughout the protein [14]. The distribution of these holes is almost homogeneous in the protein interior, provided the test volume unit is large compared to a single atom. If we choose a smaller test volume unit as is necessary for rationalizing the activation volumes, it is obvious that the holes are located at the surfaces of the atomic Van der Waal's spheres. Thus it is obvious that the space in the rotational sphere which is not taken up by the aromatic ring itself is rather empty.

To demonstrate this quantitatively in a computer calculation on the basis of the X-ray coordinates of BPTI [9], the aromatic rings of phenylalanine or tyrosine were simulated as oblate rotational ellipsoid with half-axes of 1.7 and 3.4 Å [12]. The other atoms were represented as hard spheres with radii of 1.0, 1.5, 1.35, 1.35 and 2.0 Å for H, C, N, O and CH_3, respectively, representing the minimum contact distances [15]. The centers of these spheres were placed at the positions given by the refined X-ray coordinates [9]. Proton positions were calculated from the coordinates of the non-hydrogen atoms using standard geometries. With these assumptions a simple computer algorithm was used to calculate the volume inside a sphere of 3.4 Å radius around the center of the aromatic ring which was occupied by neighbouring atoms in the single crystal conformation. This gave 16 Å^3 for Phe 45 and 20 Å^3 for Tyr 35, which is much smaller than either the experimentally determined activation volumes or the spherical volume necessary for ring rotation. Thus only a small volume has to be removed from the environment of the ring to allow a collisionless flip.

It has to be considered, however, that the atoms removed from the rotation sphere of the ring also must have a void volume at their surfaces in their new environment. Assuming the void volume for contacts between the neighbouring groups remains roughly constant, only the void volume involved in direct contact of the aromatic ring with its neighbours should be considered. These considerations suggest that the volume expansion needed for a collisionless flip lies between the 16 Å^3 or 20 Å^3 calculated from the X-ray conformation with consideration of the void volumes, and the 46 Å^3 calculated with the assumption of a homogeneously filled space in the protein interior. The large activation volumes measured are an indication that the actual ring flips occur without many collisions in an unoccupied volume provided by fluctuation of the protein conformation.

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