SINGLE MOLECULE OPTICAL TWEEZERS STUDIES OF
THE UNFOLDING/REFOLDING PROCESSES OF ACYL-
COENZYME A BINDING PROTEIN

by

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Chapter I
1. Introduction

1.1 An introduction to protein
The word protein is drawn from the Greek, meaning “primary importance”. Proteins are the basic units of life as they play a role in every part of a cell life cycle. Most of the events in living organisms are controlled by proteins such as hormones, antibodies and enzymes. DNA is transcribed into mRNA which is then translated into a polypeptide chain, Figure 1.1.

![Central dogma of molecular biology](image)

*Figure 1.1. Central dogma of molecular biology*

The newly synthesized polypeptide chain must fold into a specific three dimensional structure to generate a functional protein. If the folding process goes wrong, the protein becomes a useless and often toxic molecule for the cell. Protein folding is thus essential to life and understanding its basic rules will help us understand how living cells work.

1.1.1 Amino acids
Proteins are heteropolymer chains composed of 20 amino acids connected through peptide bonds. Each amino acid has an alpha carbon atom (C$_\alpha$), bonded to four different chemical groups (Fig.1.2). The simplest of the amino acids, glycine, has one hydrogen (H) atom as R–group (Fig.1.2). Amino acids can be classified into a few different categories based primarily on their solubility in water.
An amino acid consists of,

1. An amino group (–NH₂),
2. A carboxyl group (–COOH),
3. A hydrogen atom (–H), and
4. One variable group, called side chain or R group, which determines the chemical properties of the amino acid.

Amino acids with a hydrophilic side chain tend to be on the surface of proteins, increasing their solubility in water. Amino acids with a hydrophobic side chain tend to segregate inside the molecule generating a water-insoluble core. There are special amino acids that play special roles through the unique properties of their side chains. For example, the side chain of cysteine contains a reactive sulfhydryl group (—SH) that can react with another —SH to form a disulfide bond (—S—S—). Disulfide bonds can be formed within a single peptide chain and/or between different polypeptide chains, affecting the tertiary and quaternary structure of a protein. Not all proteins however contain disulfide bonds.

1.1.2 Peptide bond
A peptide bond is a covalent bond that links together two amino acids, (Fig 1.3).

A protein is made of a long chain of amino acids coupled together through peptide bonds. This sequence of amino acid is named “primary structure”. The repeated amide N, Cα, and carbonyl C atoms of each amino acid’s residue form the backbone of a protein from which
the various side chain groups project. At one end of the polypeptide chain there is a free unlinked amino group called the N – terminus, while at the other end there is a free carboxyl group called the C-terminus.

In early 50’s, Pauling and Corey [2-4] argued that proteins should exhibit some local ordering, that is some secondary structures. Based on energetics considerations, they predicted secondary structures that maximize the number of hydrogen bonds between the C – O and the H – N groups of the backbone, namely the alpha helix and beta sheet structures. In an alpha helix there are 3.6 amino acids per turn and the hydrogen bonds are aligned with the major axis of the helix. A β-sheet is a quasi two-dimensional structure with H-bonds oriented perpendicularly to the strands. In addition to helices and beta sheets, secondary structures may be turns or loops.

The third type of protein structure is called tertiary structure. It is the overall topology of the folded polypeptide chain. A variety of bonding interactions between the side chains of the amino acids determine this structure. Finally, the quaternary structure involves the interaction between multiple folded protein molecules.

A protein is often folded in its functional form called the native state (NS), whose entropy is considered to be zero. The denatured state of a protein often lacks a biological activity. One view of the denatured states is that of a coil state, which has no definite shape and has large conformational entropy. Another possible denatured state is the molten globule that may exist in a compact structure in low acidic conditions [5]. This state has a compact globular shape, but it does not have a well-defined structure and bears a strong resemblance to the collapsed phase of a homopolymer in a bad solvent. It is slightly less compact than the NS, and has finite conformational entropy. In vitro, the transition between the various phases is controlled by temperature, pH, and denaturant agents such as urea or guanidinium chloride.

1.2 Protein folding
Countless efforts have been carried out to study the properties of proteins and protein folding. Practically all natural proteins undergo a natural transition from a denatured state to a native state under physiological conditions. During folding, a polypeptide chain collapses into a stable three-dimensional structure [6-8]. Protein folding occurs rapidly, from μsec to sec and produces a molecule with specific biological functions. Thus protein folding enables a wide variety of biological activities that are necessary for life.
Significantly, protein folding not only aids structure production but also prevents wrong interactions between proteins. The prevention of wrong interactions is necessary in the intercellular environment where millions of proteins are present. The failure of a protein to fold into its proper structure (misfolding) can cause malfunction of vital cellular processes. To prevent these malfunctions a number of molecular systems have been developed. One of these systems is represented by molecular chaperones [9-11]. There are several diseases lumped under protein misfolding diseases, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Amyotrophic lateral sclerosis (ALS), Prion disorders, and Huntington’s disease (HD) [12]. Hence, understanding the folding mechanism is important and has attracted the attention of many researchers for over fifty years. Despite the elucidation of several universal features there are still a number of issues in protein folding still unsolved. Here we discuss the state of the art of this field.

1.2.1 Energy landscape theory
In 1958, X-ray crystallography yielded the first 3D structure of a protein, which was thought to be very rigid [13-17]. The development of other innovative experimental techniques revealed and improved our understanding of protein structure and conformational dynamics. Among the techniques that have been used for this purpose, there are nuclear magnetic resonance (NMR) spectroscopy [18-24], low-temperature flash photolysis [25-28], and hydrogen exchange (HX) techniques [29-36]. Nearly 65000 protein structures have been determined and deposited in a protein bank (www.rcsb.org). From the above experimental techniques, we now know that in their native states proteins are made upon a molecular skeleton of hydrogen-bond structural elements, \( \alpha \)-helices and \( \beta \)-sheets, which are coupled with tight turns and flexible loops.

In general, the folding of a natural protein is an intrinsic affinity process that is encoded in their primary sequence (amino acid sequence). Proteins occur in their native state only when the external conditions permit, such as pH, temperature, ionic strength, and cosolutes. In 1961, the classical experiment of Anfinsen enabled us to understand that the amino acid sequence of a protein is sufficient to determine its 3D structure [37, 38]. Soon after Anfinsen’s studies, theoreticians have clarified that the native structure of a protein represents the minimum of their global free energy landscape. This assumption has been confirmed in the majority of protein thermodynamic experiments. Based on this assumption, the transition state theory has been accepted to illustrate the thermodynamics of protein folding, such as the
equilibrium between the native and unfolded states. To date, the transition state theory has been able to successfully explain most of the observed thermodynamic experimental data. At a macroscopic level, the kinetics of protein folding can also be explained with transition state theory [39-44]. However, ensemble studies have failed to sufficiently unravel the conundrum of how a free unfolded protein efficiently reaches its native state within a biologically relevant time scale.

One of the breakthroughs in protein folding has been the theory behind Levinthal paradox, which states that a protein cannot fold by a random search through its conformational space because otherwise the whole process would take an almost infinite amount of time [45, 46]. Levinthal postulated the idea of a definite protein folding pathway that would reduce the relevant conformational space of the molecule. Thus a protein can reach its native structure within a biologically relevant time scale. This kind of conformational search draws much attention of researchers in the study and analysis of the protein folding problem. These efforts have also put focus on the role of intermediate states in protein folding.

“Every unique sequence has its own funnel” – the protein is folded to a minimum global free-energy and as said earlier, the folding process is purely dependent on amino acid sequence [47, 48]. In the “protein-folding funnel” theory [49-52], protein folding is described as a process that advances from a high free energy, i.e. highly disordered, state with few, if any,
intramolecular interactions to a low free energy state with native intramolecular interactions (bottom) (Fig.1.4). In the unfolded state, the polypeptide behaves as a random coil with maximum entropy and can sample the huge conformational space [39, 53]. In the folded state, the protein is folded into its native structure which has the relatively lowest entropy. The surface of the funnel could be smooth, but all of the current theoretical studies assume a rugged underlying free energy landscape for proteins [50, 54-56]. The rugged free energy landscape (FEL) consists of many local minima separated by barriers with different heights. Still, in the complicated FEL each protein has an available principal bowl, which can be reached within the biological time scale. One of the major challenging tasks is to understand how the polypeptide chain finds a path to its global free energy minimum in the complicated FEL. Other challenges include a description of the nature of intermediate states and the structural features of the transition state ensemble. In order to address these issues, a theory has been developed that is complementary to the “protein-folding funnel” model, that is the kinetic partitioning mechanism. According to this theory, there are many local low–energy minima located on the FEL, in which a protein is partially folded or misfolded. These local minima are demonstrated with topological frustration [53]. It is assumed that the topological frustration is an inherent property of all polypeptide chains. It is a direct consequence of the protein’s polymeric nature, as well as of the competing interactions that occur inside a protein, For example, hydrophobic residues favor the formation of compact structures while hydrophilic residues are better accommodated along extended conformations [57, 58] [59]. The fraction of molecules $\Phi$ that reaches the native bowl rapidly follows a two-state scenario without the population of any intermediates. The remaining fraction of molecules ($1 - \Phi$) reach the NS through a multi stage multipathway mechanism [60]. Experiments on hen-egg lysozyme [53], e.g., seem to support the kinetic partitioning mechanism, which is valid for folding via intermediates. This theory is widely accepted, but experimental proofs are limited. Often proteins are divided in two groups according to their folding mechanism. There are proteins that fold through intermediate states, with more than one TS’s [61, 62], and proteins that fold without intermediates, with a single TS. There seems to be also proteins that fold in a down-hill manner without any TS [63]. To conclude, protein folding studies have been carried out with a wide variety of novel experimental methods and theoretical models [33, 39, 40, 49, 54, 55, 64-72] [49-52, 54, 56] but many aspects of this process are still largely not understood.
1.2.2 Significance of the protein folding/unfolding at the single molecule level
Studying protein folding at the single molecule level enables one to monitor the folding pathways of individual molecules and go beyond the averaged information provided by bulk studies [73-75] [76-78]. In the last decade, single-molecule techniques have proven to be very powerful to study protein folding [64, 65, 73, 79, 80]. These methods allows one to follow the actual dynamics of single molecules as they undergo their transformations and present a number of advantages over more traditional bulk techniques, allowing one, among other things: i) to detect and characterize alternative, less probable folding trajectories, ii) to monitor in real time fluctuations between different molecular conformations, iii) to measure the magnitude of the forces that hold together the protein’s secondary and tertiary structures, iv) to measure directly the potential of mean force of a molecule as a function of its extension, v) to probe alternative regions of the energy landscape to those explored in experiments with thermal or chemical denaturation.

1.3 Single molecule force spectroscopy (SMFS) for protein folding
Single molecule force spectroscopy allows the study of: 1) the biophysics of heterogeneity and disorder in biological samples, 2) the precise localization and counting of molecules in a biological substrate, 3) the kinetics and statistics of complex biological processes, 4) the mechanical properties of biopolymers [81-83]. Nowadays, the most popular SMFS techniques are optical tweezers (OTs), atomic force microscopy (AFM), magnetic tweezers (MTs), and biomembrane force probe (BFP) [82, 84-86] [87-89]. Owing to their force resolution (0.1-200pN) and spacial resolution (< 1nm), optical tweezers are widely used for single molecule protein folding studies [90-92].

1.3.1 Optical tweezers (OTs)
Optical tweezers (trapping) is a powerful and versatile technique based on the interaction of light and matter. This technique is widely used in biophysics, molecular biology and biochemistry. This is an excellent technique because laser based optical traps can precisely isolate, extract, move and cut micron-sized particle such as cells, chromosomes and DNA molecules without physical contact.

The basic theory behind this technique arises from the invention of Johannes Kepler. Light exerts force on matter and this for example causes the tail of comet to always point away
from the center of its orbit, because the comet particles are pushed away from the sun’s radiation pressure. In 1873, James Maxwell’s first theory explains that the light consists of transverse electromagnetic waves [93]. Maxwell’s law shows how such waves can exert a longitudinal pushing force against matter and carry linear momentum. Later, John Henry Poynting talked out about the radiation pressure forces, during his presidential address to the British Physical Society in 1905, “A very short experience in attempting to measure these forces is sufficient to make one realize their extreme minuteness—a minuteness which appears to put them beyond consideration in terrestrial affairs …” [94]. The first successful experimental study on radiation pressure was carried out by Nichols and Hull [95, 96], and Lebedev [94]. They did experiments on macroscopic objects and absorbing gases. This subject of interest remained unimportant until the invention of laser in 1960.

The question arises with the advent of quantum mechanics: how can light exert force on matter when it possesses negligible mass (~ $10^{-51}$ g) [97]. From Einstein’s explanation of the photoelectric effect and Plank’s theory of radiation, it follows that photons, i.e. elementary light particles, are quanta of energy that possess a well-defined momentum $P = \hbar k$ (where $\hbar$ is the Plank’s constant and $k$ is the wave vector). When a stream of photons hits a dielectric object, it is either absorbed or diffracted. Because momentum is conserved, the rate of change of momentum of the light caused by its interaction with the object must be equal and opposite to the rate of change in momentum of the object. We can thus conclude that light can move, hold and exert controllable linear and angular forces on an object, depending on the physical properties of an object i.e. refractive index, absorption and size. This form of optical momentum transfer and their force is at the center of the area of optical trapping [94].

In 1968, Vladilen S. Letokhov proposed that light beams could be used to trap atoms [98] and Arthur Ashkin produced the first counter-propagating beam trap. His experiments demonstrated the effect of radiation pressure on micron-sized particles. It was observed that only high-refractive index particles are attracted into regions of high light intensity, while low index particles are always pushed out of the light beam [84]. In 1975, Hänsch and Schallow proposed that a laser could be used for atom trapping. They pointed out that it was possible to use velocity dependence of the scattering force due to Doppler shift for the optical cooling or damping of atomic motions [99]. In 1986, Ashkin and coworkers developed single beam gradient force OTs. Later Steven Chu and colleagues at Bell Laboratories were able to demonstrate and introduce three dimensional cooling techniques called “optical molasses” [99]. This technique produce a 1 cm$^3$ volume of atomic vapor at a density of $10^9$ atoms/cm$^3$, 

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viscously confined at a temperature of -250 K, which persist for times up to 1s before diffusing away. In 1987 Ashkin and Dziedzic for the first time used Nd:YAG laser to avoid optical damage to organisms, marking the first application to biological samples [100]. Since then, the OT field has developed rapidly [101].

In their first biological studies, Ashkin and coworkers used OTs to trap single cells and investigate the movement of organelles [100, 102-105].

Later, in 1989, Block used OTs to trap bacteria and measure the forces generated by their flagellum [106]. An important development was the ability to convert OTs into force transducers, with the use of back focal plane interferometry [107]. After this development, many OT experiments were carried out to study the biophysics of molecular motors [1].

A novel force measurement method was introduced by Steven Smith that developed an optical-trap force transducer that operates by direct measurement of light momentum [1]. Nowadays, force measurements using OTs has become important and widely used to study different biological systems, such as: protein and RNA folding, DNA mechanical properties, and molecular motors [108-110].

At the University of Modena and Reggio Emilia, a momentum flux force sensor dual beam OT has been developed that allows the direct measurement of light momentum, Figure 1.5. This instrument has been employed to study the folding/unfolding processes of single acyl-coenzyme A binding protein (ACBP). The following section discusses the significance of our model protein.
1.4 Brief overview of our model protein: Acyl-coenzyme A binding protein
Fatty acids are the basic root of metabolic energy, acting as storage forms of energy and as substrates for membrane biogenesis. Normally, free fatty acids pass through the cells by diffusion or by protein transport mediators, and are esterificated through acyl-coenzyme A esters by acyl-coenzyme A synthetase. Long chain acyl-coenzyme A esters act as important intermediates in fatty lipid biosynthesis and fatty acid degradation [111-117]. Also acyl-coenzyme A esters regulate lipid and energy metabolism and signal transduction. In metabolism, acyl-coenzyme A esters act as inhibitors and stimulate many enzymes. In signal transduction they are involved in regulating membrane trafficking. It was also found that acyl-coenzyme A esters are involved in gene regulation. Apart from their basic functions, acyl-coenzyme A esters require a pool performer and transporter in order to function as intermediates in lipid metabolism and as signal molecules. A molecule that achieves (in the intercellular milieu) (as a pool performer) such required functions is called Acyl-coenzyme A binding protein (ACBP) [111, 117-121].

The ACBP gene consists of 5.6 kb in humans and 8.7 kb in mouse. The ACBP gene varies from species to species and in humans the ACBP gene has three different variants [122, 123]. It was found that the human ACBP gene is responsible for many diseases associated with single nucleotide polymorphism (SNP), one of which is found in patients suffering from schizophrenia and is caused by multiple missense (stop codon) mutations in the ACBP gene [118, 119, 124]. There are 18 novel SNPs, among which three major SNPs cause significant diseases, including anxiety disorder with panic attack and type 2 diabetes. Further investigations showed that the ACBP gene is essential and necessary for biochemical functions in the cell [125-128]. The ACBP gene codes for an approximately 10 kDa protein that binds acyl-coenzyme A esters with high specificity and affinity. ACBP proteins have been extracted from yeast, plants, reptiles, humans and some resemblance has been found in pathogenic eubacterial species [129, 130].

The expression levels of ACBP vary considerably from tissue to tissue [124]. The present study focused on bovine ACBP. The amino acid sequence of ACBP is highly conserved, and consists of 86 residues folded into an up-down-down-up all α-helical bundle structure (Fig.1.6). NMR and crystal structure studies revealed the same topology in bovine, plasmodium falciparum and human ACBP [131-134]. Bovine ACBP is expressed in every tissue in eukaryotic species tested. The highest ACBP levels have been found in liver, kidney, adrenal gland, intestine and adipose tissue. In the adrenal cortex, high concentrations of
ACBP-like immunoreactivity were found in the cells of zona glomerulosa, but the levels in the zona fasciculate and reticulate were considerably lower. Also high levels of ACBP were found in the Leydig cells of testis [135-140].

Muscle tissue has relatively low expression levels of ACBP and it has been observed that ACBP levels are different depending on the muscle fiber type. The highest levels were found in slow twitch oxidative soleus muscle and lowest from fast-twitch glycolytic white gastrocnemius [141]. In the brain, the highest levels of ACBP-like immunoreactivity found in cerebellum, hypothalamus and reticular thalamic nucleus, Bergmann glia. In *Drosophila melanogaster*, ACBP has been found primarily in tissues that are associated with high energy production or fat metabolism [135, 136, 138, 142-144].

Its extensive distribution throughout the animal and plant kingdom, and its high level of sequence identity between different species suggest that ACBP is a house-keeping protein [111, 119, 123, 145, 146]. These investigations are further supported by the observation that the sequence of the ACBP gene has the characteristics of a housekeeping gene. Despite its importance as housekeeping protein in the animal and plant kingdom, the biological pathways of ACBP are not yet known entirely. In general, during biological functions protein unfolding takes places in regulation of gene, trafficking, protein degradation and quality control etc [117, 120]. Diseases associated with protein folding involve partial unfolding/folding and subsequent oligomerization. In many cases, during the folding process cytotoxic species accumulate in an intermediate state before aggregation. In order to understand these processes, a detailed study of the protein folding mechanism is crucial.

Traditionally, the folding of ACBP has been investigated extensively using bulk methods such as stopped flow method, circular dichroism (CD), mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. From these spectroscopic studies, it has been observed that ACBP is a fast folder which folds and unfolds in a two-state fashion, with no indication of partially structured intermediates. The two-state folding behaviour is apparently encoded within the sequence of ACBP. In order to analyze the role of conserved residues in the folding of ACBP, a number of single-amino acid mutants of ACBP were analyzed by kinetic chevron plots [111, 147]. It was observed that the interference of helices had a large impact on the folding rate and that many residues are likely to form favorable native-like interactions in the rate determining folding step [147].
The aim of the present study was to provide new insights into the unfolding/refolding mechanism of ACBP through single molecule optical tweezers studies.
Chapter II
2 Optical tweezers—Experimental setup
In this chapter I will explain the physics of optical trapping and provide details on the optical tweezers setup built at the Physics Department of the University of Modena and Reggio Emilia.

2.1 Physics of optical trap
In 1986, Askin and co-workers [148] demonstrated that, by focusing a laser beam through a microscope objective, tiny objects of high refractive index, such as plastic beads or oil droplets, could be trapped and manipulated. This single-beam trapping device was termed “laser tweezers”. When the dimensions of the trapped object are much greater than the wavelength of light, the mechanism by which light traps particles can be explained by a simple ray optics model. Rays of light carry momentum and are either absorbed or diffracted when hitting dielectric objects, whose refractive index, $n$, is greater than that of the surrounding medium. Because momentum is conserved, the rate of change of momentum of the light caused by its interaction with the object must be equal and opposite to the rate of change in momentum of the object. If a particle acts as a positive lens and refracts light towards its center, then that particle can be entrained in a light beam. Consider a collimated beam of light with a Gaussian intensity profile hitting a refractive sphere that is situated off-axis in the beam, as shown in (Fig. 2.1, i). For each ray in the beam the components of momentum flux parallel and perpendicular to the optical axis are given bellow.

\[
\frac{dp}{dt}\parallel = n_B (W/c) \cos \theta \\
\frac{dp}{dt}\perp = n_B (W/c) \sin \theta
\]

Where $W$ is the power of the light ray, $\theta$ is the angle of the ray to the optical axis, $c$ is the speed of light, and $n_B$ is the refractive index of the liquid surrounding the sphere [149].

The reaction impulse ($F$) felt by the bead is equal but opposite to the change in the light momentum flux summed over all rays passing through the bead is

\[
F_{\text{bead}} = -\sum_{\text{rays}} (dp/dt)_{\text{in}} - (dp/dt)_{\text{out}}
\]
If the bead hit by the beam of light acts as a converging lens, as shown in (Fig. 2.1, i), then the strong central rays are refracted away from the beam axis, while the weaker peripheral rays are refracted towards it. This results in a net downward change of light momentum flux that causes the bead to feel a force that draws it towards the center of the beam. However, since the parallel component of the ray momentum is reduced after refraction, the bead feels an additional scattering force that pushes it downstream. Additionally, the bead is scattered forward by reflected rays. Thus, when a particle is hit by a collimated beam of light with an intensity profile peaked in its center, the particle is drawn into the intense part of the light beam, but it is also propelled away from the light source [1].

\[
\text{Gradient force}
\]

(i)

![Figure 2.1: Light momentum effects on a refractive sphere. i) A collimated laser beam with a Gaussian intensity profile entrains a bead, but it also pushes it away from the light source. ii) A focused laser beam with a Gaussian intensity profile traps a bead at a particular point in space. The scattering force caused by reflected rays is balanced by the refraction of the high angle rays and the particle is trapped slightly off center.}
Dual beam optical trap

Figure 2.2: Dual counter-propagating laser beams. i) In a dual-beam trap, the scattering forces caused by reflected rays are canceled, while the gradient forces are doubled; the bead is trapped at the light focus. ii) Light-momentum force sensor, based on (Fig. 2.1)[1]. Two low numerical aperture (NA) beams are used with two high-NA objectives. Each objective is used twice: to focus one beam and collect the other beam for analysis. The narrow cones of light used in this set-up allow the collection of nearly all the light leaving the trap, even after significant deflection of the beam. When an external force is applied on the bead, the bead moves slightly downward and the light is refracted asymmetrically. By collecting all the light momentum leaving the trap it is possible to measure the force acting on the bead as $F = (W/c)(x/RL)$, where $W$ is the light intensity, $c$ is the speed of light, $x$ is the offset measured by the photodetector and $RL$ is the focal length of the lens [1].
In order to trap a bead at a particular point in space, the beam of light must be focused (Fig. 2.1, ii). In this situation, a bead at the focus is pushed forward by the reflected rays until the scattering force is compensated by a backward force caused by the increase in the forward momentum flux of the rays refracted by the bead. The bead is thus trapped slightly downstream of the light focus. The larger the focal cone angle of the beam is, the stronger the backward force that opposes the axial escape of the bead is. Thus, to efficiently trap a bead with a single-beam trap, the back aperture of a high numerical aperture (NA) objective must be completely filled by the trapping beam.

In order to trap the bead at the light focus and avoid the use of large focal cone angles, dual counter-propagating laser beams with a common focal point must be used, as shown in (Fig. 2.2, i). In this experimental set-up, the scattering forces generated by reflected rays are canceled, while the transverse gradient forces are doubled. With this configuration it is possible to use low NA beams with high NA objectives, and determine the force applied on a trapped object by measuring directly the change in momentum flux of the trapping beam [1], (Fig. 2.2, ii).

2.2 Optical tweezers setup

Figure 2.3 shows a schematic drawing of the force measuring dual beam optical tweezers setup that we built at the Department of Physics of the University of Modena and Reggio Emilia. The optical trap is generated using two single mode counter propagating 845 nm diode lasers (Lumics) that can generate up to 150 mW light power. Two laser drivers control the intensity of the laser beams, while two temperature controllers keep each laser at optimal operating conditions. For simplicity, we will describe only the optical path of one laser, as the other one is just its mirror image. The laser optical fiber is connected to a single lens fiber port that collimates the light into a ~ 1.6 mm laser beam. Any distortion of the linear polarization of the light caused by bending of the optical fiber is corrected through a quarter-wave plate placed downstream of the collimating fiber port. Downstream of the quarter-wave there is a half-wave plate that rotates the linear polarization of the light to let the beam enter a Faraday optical isolator that protects the laser diode from any reflected ray. The linear polarization of the isolator output light is then rotated by a half-wave plate to be perpendicular to the optical table, and the laser beam is expanded two fold by a beam
expander to a diameter of ~ 3.2 mm. This laser beam underfills the objective back aperture (~ 8.0 mm), allowing almost all the light leaving the optical trap to be collected even after significant deflection of the beam. This permits us to determine the force applied on a trapped bead by measuring directly the change in momentum flux of the trapping beam.

The 3.2 mm laser beam then hits a polarizing beam splitter (objective pbs) that directs it towards a quarter-wave plate (qwp) and a microscope objective lens (OBJ, Nikon Plan Apo VC, 60x, 1.2 NA), which focuses the beam into a spot where a bead is trapped. The quarter-wave plate ensures that the light rays reflected by the trapped particle do not return to the laser. The beam exiting the optical trap is collected by the opposite objective, converted to horizontal-polarized light and directed to position-sensitive photodetectors (PSD) by a second polarizing beam splitter (detector pbs). Since the PSDs cannot be placed at the output principal plane of the objective, which lies inside the objective, a relay lens is used to re-image the principal plane onto the photo-detectors. The detectors measure only the light exiting the optical trap, not entering it, so each one performs only half of the integration required in Eq. (8). This problem is solved by aligning the detectors on the optical axis so that when no bead is present in the trap, the output beams are centered on the detectors and the difference signals vanish, corresponding to zero volts at outputs Dx and Dy. The light entering the trap carries no transverse momentum in this frame of reference and need not be considered even after a particle has been introduced. Only the exiting light is affected by interaction with the particle [1]. Two position sensitive photo-detectors are use at each side of the counter-propagating dual beam optical tweezers, one for the x and y directions and the other one for the z-direction. A 50:50 non-polarizing beam splitter equally divides the light coming out from the optical trap to these photo-detectors. A transmission filter is placed before the z-direction photo-detector in order to make the signal voltage output proportional to the z-force component.

During the experiment, individual molecules are manipulated using a polystyrene bead held in place at the end of a micropipette by suction, and another bead held in the optical trap, (Fig. 2.4). The molecule is stretched and relaxed multiple times by moving the fluid chamber, and thus the micropipette, relative to the optical trap. The movement of the fluid chamber is controlled by a piezoelectric actuator, (Fig 2.3). The changes in the extension of the molecule were measured by the movement of the two beads. The bead on the micropipette was monitored by a “light lever” system that monitors the position of the fluid chamber (Fig 2.4, 2.8). During the measurements, the beads and the micropipette are visualized using a light-
emitting diode (LED) ($\lambda = 465$ nm) and a charge-coupled-device (CCD) camera. Figure 2.3 shows a picture of the optical tweezers setup that we developed in our laboratory at Modena.

Figure 2.3: Schematic of the dual counter-propagating laser beam optical tweezers setup built in Modena.
Figure 2.4: Light lever system
2.3 Fluid Chamber

2.3.1 Making fluid chambers
In our laboratory we are now able to make the fluid chambers and the micropipettes used for our experiments.

The first step for making a fluid chamber is to drill holes in a coverslip (VWR Scientific, #2, 24mm x 60mm x 0.2mm thick). In order to accomplish this we utilized an engraver (Epilog, model Mini 18x12, 25W) that uses a 25W CO$_2$ laser as the engraving source. The pattern for drilling the holes is shown in Fig. 2.6

![Pattern utilized to drill holes in the coverslip using the engraver. Drawing is not to scale.](image)
The second step is to engrave the pattern shown in Fig. 2.7 in the Nescofilm®.

*Figure 2.7: Pattern for engraving in the Nescofilm®.*

After drilling the holes in the coverslip and engraving the pattern shown in Fig. 2.7 in the Nescofilm® we start assembling the fluid chamber. First, we take one drilled coverslip and lay down on top of that one engraved Nescofilm®, taking care to match up the holes of both coverslip and Nescofilm®. Then we place the bearing tube which will be used later as a guide to introduce the micropipette into the fluid chamber. Then we place the dispenser tubes obliquely, one in the upper and the other in the lower channel. On top of all this we add another patterned Nescofilm® taking care again to match the patterns. Finally we place another not drilled coverslip. The final step is to seal the fluid chamber and to accomplish that we placed the fluid chamber between two hot plates both at 120 C for 5 minutes. This period of time usually is enough to melt the Nescofilm® producing then an excellent seal. Fig. 2.8 shows a picture of the fluid chamber after the sealing step.

*Figure 2.8: The fluid chamber.*
In our laboratory we also assembled a pipette puller to make the micropipettes used in our experiments. Figure 2.9 shows a picture of the pipette puller apparatus that works in the following way. We introduce a glass tube (Garner Glass Company, Kimax glass tube ID = 40 µm, OD = 80 µm, Length = 150 mm) inside an iridium wire coil and keep the glass in place using the clamps shown in Fig. 2.9. Then we switch on the current ramp generator that increases the current passing through the iridium wire until it reaches 5 A. At this point the iridium wire becomes incandescent and delivers enough thermal energy to melt the glass tube. When this happens, the sliding weight to which the glass is clamped slides down and a new micropipette is produced.

![Pipette Puller and Current Ramp Generator](image)

*Figure 2.9: Pipette puller and current ramp generator source.*
2.4 Calibrations

2.4.1 Light momentum
Let’s consider now the total variation of light momentum flux caused by a force $F$ applied on a trapped particle. This force can be written in terms of the Poynting’s vector as

$$\vec{F} = \left( \frac{d\vec{P}_m}{dt} - \frac{d\vec{P}_{out}}{dt} \right) = -\frac{n_B}{c} \int \left( \vec{S}_{in} - \vec{S}_{out} \right) dA$$  \hspace{1cm} (4)

Where $\vec{S}$ Poynting’s vector and $dA$ is an element of area normal to $\vec{S}$. For an optical trap, integration can be performed over a distant ($R \gg \lambda$) spherical surface centered on the focal point. Here $\vec{S}_{in}$ is normal to the surface because the incoming wave is spherical, and $\vec{S}_{out}$ is normal to the surface because it emanates from a point (or object) at the trap. Because the radius of the sphere, $R$, is arbitrary, we can define an angular intensity distribution for light entering or leaving the focus, $I(\theta, \phi) r d\gamma = \vec{S} dA$. Although it is difficult to predict $I(\theta, \phi)$ for light scattered from an arbitrary object trapped at focus, it is not difficult to measure $I(\theta, \phi)$. A convenient way to measure it is afforded by a version of the Abbe sine condition, which states that any ray emanating from the principal focus of a coma-free objective lens, inclined at an angle $\theta$ to the optical axis but still hitting the lens, will exit the image-side principal plane of that lens at a radial distance $r$ from the optic axis given by [1]

$$r = R_L n_B \sin \theta$$  \hspace{1cm} (5)

Here $R_L$ is an effective radius for the lens that is equal to its focal length.

If the rays exiting the trap in a small element of solid angle $d\gamma / 4\pi$ are projected without loss onto an area element $dA' = rd\phi dr$ on the image-side principal plane of the lens, then by energy conservation, the irradiance $E$ (in watts/m$^2$) on $dA'$ is given by $E(r, \phi) dA' = I(\theta, \phi) d\gamma / 4\pi$. If the lenses intercept all the light exiting the trap, then equation (4) can be written as:

$$\vec{F} = \frac{1}{c} \int \int E(r, \phi) \left( \hat{i} \frac{r}{R_L} \cos \phi + \hat{j} \frac{r}{R_L} \sin \phi + \hat{k} \sqrt{n_B^2 - \frac{r^2}{R_L^2}} \right) rd\phi dr$$  \hspace{1cm} (6)
Where the integral is now taken over the surface of the image-side principal planes of the objectives [1].

The transverse ($\hat{i}$ and $\hat{j}$) components of the force can be integrated by placing position-sensitive photodetectors at that principal axis. A dual-axis detector of that type gives two differences signals, $D_x$ and $D_y$, each proportional to the detector’s responsivity $\Psi$, and to the sum of local irradiances $E(x,y)$ weighted by their relative distances $x/R_D$ or $y/R_D$ from the detector center, where $R_D$ is the detector’s half-width. $D_x$ and $D_y$ are given by the following expressions:

$$D_x = \Psi \int \int E(r,\phi)(r \cos \phi / R_D) dA'$$
$$D_y = \Psi \int \int E(r,\phi)(r \sin \phi / R_D) dA'$$

(7)

By combining equations (6) and (7), expressions for the two components of force transverse to the optical axis, $F_x$ and $F_y$, can be obtained in terms of the detector signals and known constants [1]

$$F_x = \frac{D_x R_D}{c\Psi R_L}$$
$$F_y = \frac{D_y R_D}{c\Psi R_L}$$

(8)

From equations (8) we can see that the measurement of force exerted on the trapped particle does not depend on any information about the particle size or shape but only relies on the instrument parameters $R_D$, $R_L$, and $\Psi$ that do not vary with the experimental conditions. In our optical tweezers, equations (8) are used to determine the forces applied on the trapped particles along the x- and y-axes. The force applied on the particle along the optical axis (z-axis) is described later in this chapter.

Thus, to calibrate our optical tweezers set-up we only need to determine the values for the following parameters: $R_D$ or the effective radius of our PSDs, $R_L$ or the focal length of our objectives and $\Psi$ or the sensitivity of our PSDs. To determine $R_D$ we removed both objectives and the fluid chamber and then we placed a diode laser mounted on a mechanical translator stage at the same place where the fluid chamber was; initially the laser beam should hit the center of one of the PSDs. We varied the position of the laser beam by moving the
translation stage while recording the output from the PSD. From the graph of output PSD voltage as a function of relative position we were able to determine $R_D$.

After determining $R_D$ we re-assembled the objectives and the fluid chamber. The sensitivity $\Psi$ of the PSDs can be obtained by measuring the intensity of the laser beam before entering and after leaving the objectives. We can assume that a good approximation for the intensity of the laser beam at the focus point is the geometrical mean of those two readings [1]. Then $\Psi$ is the ratio between the voltage measured by the PSD and the laser intensity at the focus. We must remember that this measurement should be done with one laser running at a time.

The last parameter $R_L = 200/60 = 3.33$ mm is the focal length of our objective. Using these parameters we obtained a force calibration factor of 109.844608 pN/V.

### 2.4.2 Stokes drag force

The Stokes drag force method was utilized to check the calibration of the force sensor using the methodology described in previous section. The Stokes drag force calibration method consists in measuring the applied force on a trapped bead that is subject to a laminar flow inside the fluid chamber [1]. The laminar flow inside the fluid chamber was obtained by translating back and forth by hand the piezo stage at different speeds. The acquisition program is set in such a way that it collects data only when the fluid chamber velocity is almost constant (within 10%). Figure 2.10 shows the results obtained over a range of velocities with a 2.1 µm bead. The measurements were performed far from the coverglass surfaces to avoid any corrections due to Faxen’s law [150, 151]. The fluid chamber position was determined by an electronic micrometer attached to the piezo stage. In principle, within the experimental error, the force measured by

$$F = 6\pi \eta r v = \xi v,$$

Where $\eta$ is the viscosity of the liquid inside the fluid chamber which we consider being water, $r$ is the radius of the particle, $\xi$ is the coefficient of viscous drag and $v$ is the velocity of the fluid flow inside the chamber; here there is the assumption that the velocity of the liquid is the same of that of the fluid chamber.
Figure 2.10: Force applied on the trapped bead measured by the light momentum principle as a function of the velocity of the liquid flow. The slope of the linear fit for 2.1 µm bead is -0.0164 pN s/µm and the slope of linear fit for 3.18 µm bead is -0.02577 pN s/µm. The temperature of the fluid chamber was 22.7°C measured using a K-type thermocouple.

For a 2.1 µm bead the value of \( \frac{\xi}{6\eta} = 0.018456 \) pN s/µm when the fluid chamber is at a temperature of 23°C. The value that we obtained from (Fig. 2.10) is around 10% of the value that we got using the light momentum method; this is a reasonable value if we consider that in our measurements we have some uncertainties, like for example the actual radius of the bead that we consider to be equal to the nominal value provided by the manufacturer. This discrepancy could also be due to the shape of the bead not being spherical but instead slightly ellipsoidal. We also performed the same measurement for 10 different 2.1 µm beads and obtained slope values within 3% to the one shown in (Fig. 2.10). For 3.18 µm beads we also obtained slope values within 10% (Fig. 2.10) of the \( \xi = 0.02795 \) pN.s/µm value.

### 2.4.3 Thermal fluctuation

Another way to check the calibration of our optical tweezers force sensor is to measure the thermal forces (Brownian motion) acting on a trapped bead and compare the measured value with theory. The equation of motion of a trapped bead can be expressed as:

\[
\xi \frac{dx}{dt} + kx = F(t)
\]

(10)
Where $\xi$ is the coefficient of viscous drag, $k$ is the spring constant of the optical trap. If $F(t)$ is due to thermal fluctuations, it is random and has an average value of zero and its power spectrum is constant, such that:

$$\langle \Delta F^2(\omega) \rangle_{eq} = 4\xi k_B T$$

(11)

Where $k_B$ is the Boltzmann constant and $T$ is the temperature. The spectral density of force fluctuations is then given by:

$$\langle \Delta F^2(\omega) \rangle_{eq} = 4\xi k_B T \frac{\omega_c}{\omega_c^2 + \omega^2}$$

(12)

Where $\omega_c$ is the corner frequency and is related to the spring constant $k$ and the coefficient of viscous drag by:

$$\omega_c = \frac{k}{\xi}$$

(13)

To perform the calibration with thermal force, we recorded the force fluctuations acting on a trapped bead utilizing a fast A/D (analog-to-digital) converter from National Instruments (PCIe 6259 with 16-bit accuracy) with a sampling rate of 100 kHz and acquisition time equal to 21 s. The time interval was then split into 256 parts of 82.03 ms. Next the Fourier transform of force fluctuations was calculated and averaged over the 256 samples. The sampling at 100 kHz sets a maximum usable frequency of 50 kHz (Nyquist frequency $f_{Nyq} \equiv \frac{f_{sample}}{2}$). For a 2.1 $\mu$m bead the corner frequency measured was 1106.2 Hz (Fig. 2.11).

From this value we calculated the trap stiffness $k$ (spring constant) using Equation (13) and we got a $k$ of 0.128 pN/nm.

Since we have an increase in the noise in the low frequency region, below 400 Hz, we decided to perform the same kind of measurements at the lower sampling rate of 10 kHz during 100 s; also in this case we used the same procedure for averaging the Fourier transform. By doing this we hoped to have less noise in the low frequency region in order to be able to extrapolate and obtain the value of the plateau $\langle \Delta F^2(\omega) \rangle_{eq}$, which is used to compare the two calibration methods. Figure 2.12 shows the result that we obtained when we sampled the trapped bead at 10 kHz for 100 s. As we can observe the noise level only
increases for frequencies below 40 Hz, allowing us to confidently determine the $\langle \Delta F^2(\omega) \rangle_{eq}$ value by extrapolation. In the case of a 2.1 µm bead the extrapolated value of $\langle \Delta F^2(\omega) \rangle_{eq}$ obtained from (Fig. 2.12) is $2.47 \times 10^{-4}$ pN$^2$/Hz. Since there is a way to compare this experimental value with the expected one by using Equation (11), we obtained $\langle \Delta F^2(\omega) \rangle_{eq} = 4\xi k_B T = 2.764 \times 10^{-4}$ pN$^2$/Hz, considering that the temperature of the fluid chamber was 25 C. Thus we have a good agreement between both values of $\langle \Delta F^2(\omega) \rangle_{eq}$ (within 10%) demonstrating that the calibration performed by first principles in the case of light momentum calibration indeed worked well in our optical tweezers setup.

Figure 2.11: Power spectral density distribution of force fluctuations from light-momentum force sensor using a 2.1 µm in diameter polystyrene bead. One hundred seconds of output was recorded at a 10 kHz rate using a National Instruments PCIe 6259 analog-to-digital converter with 16-bit accuracy. The corner frequency is $\omega_C = 1105.1$ Hz.
2.4.4 DNA overstretching transition

After knowing that the three force calibration methods agree reasonably well with each other (within 10%) we decided to pull on a double-stranded DNA molecule.

Figure 2.13: Force-extension curves for a double-stranded 2.5-kbp DNA molecule pulled in 250 mM NaCl, 10 mM Tris, 10 mM MgCl$_2$, pH 7.0. The DNA overstretching transition is observed at ~ 67 pN.
We decided to perform this experiment because it is known that when a double-stranded DNA molecule is stretched it undergoes a transition from B- to S-form at about 67 pN of force [152, 153]. Figure 2.13 presents two stretch-relaxation cycles obtained by pulling on a 2.5-kbp double-stranded DNA. Under our experimental conditions the DNA overstretches at about 67 pN, which is a force value very close to the one previously published (67 pN value). This result shows that the calibration in force of our instrument is good.

### 2.4.5 Position calibration

The calibration for position measurement was performed by moving the micropipette by a known distance in two different directions $x$ (horizontal) and $y$ (vertical). We need to perform the calibration in both directions because the size of the panel where the image of the fluid chamber is displayed is 640 x 480 pixels.

Figure 2.14, i and 2.14, ii show the experimental data obtained for the calibration of the image acquisition system and the corresponding linear fitting curves.

![Figure 2.14](image)

**Figure 2.14:** (i) Vertical calibration of the image acquisition system. The mean calibration factor is 7.63705 pixels/$\mu$m, (ii) Horizontal calibration of the image acquisition system. The mean calibration factor is 5.98835 pixels/$\mu$m.

### 2.4.6 Z-axis force measurement

The $x$ and $y$-axis force measurements are straightforward as one can see from Equation (6). Once we utilize PSDs we can determine the externally applied force on a trapped bead by measuring the displacement of the laser over the photodetectors surface in relation to the center of the detector and then convert this value to force by a conversion factor as discussed.
in section (2.4.1). Instead for the \( z \)-component of the force the measurement is not as straightforward as shown by Equation (14):

\[
F_z = \frac{1}{c} \iint E(r, \phi) \sqrt{n_i^2 - \frac{r^2}{R_L^2}} \, rdrd\phi
\]

(14)

For the \( z \)-component of the force we need to modulate the light intensity in order to use the signal from the photodetectors as a measure of force. To accomplish this we had to design a transmission filter whose transmission pattern follows the relation:

\[
T = \sqrt{n_i^2 - \frac{r^2}{R_L^2}} = \sqrt{1 - \left( \frac{r}{n_i R_L} \right)^2}
\]

(15)

Equation 15 tells us that the transmission should decrease as we get far from the center of the attenuator.

We designed the \( z \)-axis transmission filter by using MatLab. Figure 2.15 shows the final drawing of the transmission filter. You can see that we introduced some guidelines in order to make easier the alignment of this filter in front of the \( z \)-axis photodetector. We should keep in mind that the filter should be in the same optical axis as the photodetector. There is also a square shaped black region surrounding the circular transmission filter which is supposed to prevent any stray light from passing through the edges of the filter and hitting the photodetectors.

Figure 2.15: Final drawing of the transmission filter. Red arrow indicates the circular shaped transmission filter (whose transmission pattern follows Equation (15)) surrounded by a square black region that should prevent light from going through the edges of the filter.
The pattern shown in Figure 2.15 was then printed in a plastic transparency sheet using a high resolution laser printer. The optical quality of the plastic transparency sheet was a concern since any opacity could compromise the final quality of the transmission filter. We utilized 3M model CG3300 transparency sheets. In order to assure that the transmission filter has the expected transmission profile we performed transmission measurements by utilizing a 1mW diode laser (beam diameter 0.5 mm) and a power meter (Newport model 841-PE + detector head model 818-SL). The diode laser scanned the transmission filter in two perpendicular directions while the transmittance was recorded by the power meter. Figure 2.15 shows the transmittance of the filter versus the position of the laser spot on the filter’s surface. The experimental data are represented by black squares, while the fitting curve is shown in red. The fit of the experimental data was performed using

\[
T = P_2 \sqrt{1 - \left( \frac{x - P_3}{P_1} \right)^2},
\]

(16)

Where \( P_1, P_2 \) and \( P_3 \) are the fitting parameters, \( P_2 \) and \( P_3 \) are necessary for offset adjustments. The best fit (least squares) values were:

\[
\begin{align*}
P_1 &= 4.45 \\
P_2 &= 53.2 \\
P_3 &= 6.24
\end{align*}
\]

Figure 2.16: Transmission profile of the z-axis filter. Black squares are experimental data acquired by measuring the transmittance of the filter shown in Figure 2.15. Red line is the curve obtained after fitting the experimental data with Equation (16).
Comparing equations 15 with equation 16 we see that $P_1$ should be equal to $n_1 R_L$. In our setup $n_1 = 1.334$ and $R_L = \frac{200}{60} = 3.33$, thus the product of these factors equals 4.44. The value that we measured for $P_1$ is very close to the expected one, showing that the transmission profile follows Equation (15) well.

2.4.7 Force resolution
An important issue in our OTs was its force resolution. To determine this value we performed the following experiment. First we aligned and focused both beams at the same spot inside the fluid chamber, and then we moved the micropipette inside the optical trap until we got a certain force value, then we set the control program to keep the force constant and we recorded the force value as a function of time. We did these measurements at different forces. Figure 2.17 shows the results of these measurements. The upper graph shows the force measured as a function of time, while the lower graph shows the piezoelectric stage position (which in fact is the position of the micropipette) as a function of time. From these data we learned that the force resolution of our equipment is 0.1 pN since, in the upper graph, we can clearly resolve the 0.1 pN steps. The spikes seen in this graph are due to air movements produced by opening/closing doors outside our laboratory. The lower graph shows the movement of the piezoelectric stage to keep the force constant.

![Figure 2.17](image)

*Figure 2.17: The upper panel shows the force as a function of time, as the force is kept constant by our algorithm. The lower panel shows the piezoelectric stage position as a function of time.*
Chapter III
3. Experimental materials and methods

3.1 Introduction
A major issue in single molecule manipulation studies is to find conditions that facilitate the attachment of the molecule under study to movable substrates, while keeping the strength of the interactions between the tethering surfaces to a minimum. This is a difficult task to fulfill when trying to manipulate nanometer-sized globular proteins with micrometer-sized beads, such those used in laser tweezer experiments. A direct attachment of the protein molecule to the beads would in fact require the large tethering surfaces to come so close to each other they would interact, thus compromising the measurements. To overcome this problem, Cecconi and co-authors developed a new experimental procedure to make globular proteins amenable to mechanical manipulation in laser tweezer experiments [154]. This approach relies on the use of molecular handles, ~500 bp DNA molecules, to specifically connect the protein to polystyrene beads and keep the attachment points at a distance at which unspecific interactions between the tethering surfaces are negligible. One end of each DNA molecule is covalently attached to a cysteine residue through a disulfide bond, while the other end is bound to a bead through either streptavidin-biotin or digoxigenin-antibodies interactions.

3.2 Synthesis of molecular constructs for dual beam optical tweezers

3.2.1 Preparation of ACBP
Cysteine-modified ACBP were generated using the Quick-Change Site-Directed mutagenesis kit from Stratagene. All genes were cloned into high copy plasmids under a T7 promoter (pET3a). ACBP was purified using previously published protocols with the difference that 1 mM DTT was present during all purification steps [155-157].

3.2.2 Gel Electrophoresis
Linear polyacrylamide SDS gels were prepared using a Bio-Red apparatus. Gradient polyacrylamide SDS gels, 4-20%, were purchased from Bio-Red. Gels were silver stained to identify the reaction products.
3.2.3 Atomic force microscopy
AFM was performed in air with a Digital Instrument Nanoscope III, in tapping mode, using tips from Nanosensors (pointprobes, type NCH-100). Molecular constructs were diluted to a final concentration of 2 nM in deposition buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 2 mM MgCl$_2$). Twenty microliters of this solution were deposited onto freshly cleaved mica (Asheville-Schoonmaker Mica Company #472X8 /10XSF) and allowed to adsorb onto the surface for 1 minute. The sample was then gently washed with doubly distilled water and dried with a stream of nitrogen.

3.2.4 DTDP activation of cysteine-modified ACBP
A 500 µM solution of ACBP in buffer A (0.1 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0) was typically reduced with 10 mM DTT for ~ 1 hour at RT, and then buffer exchanged into 0.1 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 5.5 using size-exclusion columns, either Sephadex G-25 (Pierce) or HiTrap desalting columns (Amersham Biosciences). The resulting reduced ACBP was reacted with a concentrated stock of DTDP (4.5 or 10 mM in 0.1 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 5.5, 15% acetonitrile) such that DTDP was in 5- to 25-fold molar excess of the protein, and allowed to react for 2–24 h at RT. The excess of DTDP was then removed using size-exclusion columns equilibrated with buffer A.

3.2.5 Generation of DNA handles
The 558 bp DNA handles were generated in large quantities by PCR using Taq DNA polymerase and pGEMEX 1 plasmid DNA from Promega as template. Usually 400–500 µg of handles were generated at a time using 9.6 ml of PCR reaction. The two types of handles were generated using the primer 5´ thiol-GCT-ACC-GTA-ATT-GAG-ACC-AC together with either the primer 5´ biotin-CAA-AAA-ACC-CCT-CAA-GAC-CC or the primer 5´ digoxigenin-CAA-AAA-ACC-CCT-CAA-GAC-CC. The PCR products were purified using HiSpeed Plasmid Maxi Kit, from QIAGEN.

3.2.6 Attachment of DNA molecules to single ACBP domains
In order to obtain samples with the ability to bind differentially to two functionally diverse polystyrene beads (specifically, a bead coated with anti-digoxigenin antibodies and another coated with streptavidin), two distinctly modified 558 bp dsDNA handles were attached to the ACBP molecules. One handle contained a 5´ thiol group and a 5´ biotin moiety; the other
handle contained a 5´ thiol group and a 5´ digoxigenin moiety. The attachment of the two handles was carried out as described below through a one-step chemical reaction (Fig. 3.1).

**A)** Attachment of DNA handles to protein molecule

\[
2 \text{ DTDP} + \text{Cysteine-modified} \rightarrow \text{Thiol-pyridine-activated}
\]

**B)** Attachment of DNA molecules to activated protein thiols

\[
\text{DNA} + \text{Activated protein thiol} \rightarrow \text{DNA bound to activated protein thiol}
\]

**C)** Attachment of 558 bp DNA handles to protein molecules

Figure 3.1: Schematic of the chemical reaction used to attach DNA to ACBP molecules, (A) DTDP activation of cysteine-modified ACBP. (B) DNA protein coupling. (C) Atomic force microscopy images (i). DNA handles alone, (ii). ACBP bound to one handles, (iii). ACBP bound to two handles. (iv) 7.5% SDS-PAGE gel of handles alone (DNA) and product of one step attachment (DNA+ACBP).

### 3.2.7 One-step attachment

Handles can be attached to ACBP molecules through a simple one-step reaction. In this case, a thiol-pyridine activated protein was reacted with a two to fourfold excess of a 1:1 mixture of the two handles at RT for 12–24 hours. The final result is a mixture of DNA modified...
proteins where only 50% of the molecules have the required configuration of one biotin- and one digoxigenin- labeled handle. As expected, the time course of this reaction is biphasic; a fast phase occurring during the first 20–30 minutes, probably corresponding to the attachment of the protein to one handle, is followed by a much slower phase that likely corresponds to the attachment of the second DNA molecule (data not shown). This method provides a good yield of correctly labeled proteins (ACBP) and has been our method of choice to prepare samples for the optical tweezers.

The one-step attachment protocol does not require any further purification, as only correctly labeled proteins will function in the optical tweezers experiments. Other species present in the sample, such as protein without/with one handle or protein flanked by identical handles, are unable to bind simultaneously to the two differently functionalized beads and therefore do not complicate our data. Sporadically, force-extension curves lacking any discontinuity were observed; these traces likely come from the stretching of DNA–DNA dimers.

### 3.2.8 Attachment of ACBP-DNA chimeras to polystyrene beads

ACBP-DNA constructs were first reacted with polystyrene beads coated with anti-digoxigenin antibodies (dig-beads) for 5–15 minutes at room temperature (RT) (Fig. 3.2). Dig-beads were generated by coupling anti-digoxigenin antibodies (Roche) to 3.18 µm proteinG-coated beads (Spherotech). After reacting with the ACBP-DNA construct, a dig-bead was placed in the optical trap (common foci of two objectives) and then brought in close proximity to a 2.10 µm streptavidin-coated bead (Spherotech) which was held in place at the end of a pipette by suction, until a tether between the two beads was attained (Fig. 3.3) [158].
Figure 3.2: Schematic of the experimental procedure used to tether single proteins in optical tweezers experiments. A) Polystyrene beads covered with antibodies against digoxigenin are first allowed to react with the DNA-protein chimeras for 15 minutes at RT. B) The DNA-protein chimera beads are then flown into the fluid chamber of the optical tweezer instrument and caught in the optical trap. A second bead covered with streptavidin was previously attached to a micropipette by suction. This streptavidin bead is then brought close to the optical trap bead to facilitate its binding to the DNA biotin moiety on the free end of the DNA-protein chimera.

3.3 Mechanical force manipulation of DNA-modified ACBP using the dual beam optical tweezers
ACBP coupled to long DNA handles was manipulated using the optical tweezers set-up depicted in (Fig. 3.3). During the experiment, ACBP is stretched and relaxed by moving the micropipette relative to optical trap. The force applied on the molecule is measured by monitoring the change in light momentum flux of the light beams leaving the trap [1]. The changes in the extension of the molecule is measured by tracking the movement of the two beads and using a “light lever” system that monitors the position of the fluid chamber, see chapter 2. The force–extension curves for DNA have been well characterized [152, 153, 159], therefore the contribution of the DNA handles to the overall signal can be easily distinguished. Handle fluctuations can sometimes give rise to small transitions in the observed traces. These transitions can be carefully characterized with control experiments,
where handles alone are pulled, and easily distinguished in the overall signal. Figure 3.4 shows a typical force–extension curve obtained by pulling on a single ACBP molecule.

Figure 3.3: Schematic sketch of the experimental set-up used to manipulate single ACBP molecule by dual beam optical set-up.

Three types of mechanical manipulation experiments were carried out in the present study that is, force-ramp, force-clamp, and force-jump experiments. During force-ramp experiments, the molecule is stretched and relaxed multiple times by moving the piezo stage at a constant speed (nm/s) in one dimension. This generated a constant loading and unloading rate of pulling (pN/s) [83, 153, 160, 161].
In constant force experiments, both force-clamp and force-jump, the force acting on the molecule is kept constant through a feedback mechanism that programmatically moves the micropipette up or down with a piezoelectric actuator \cite{162, 163}. When the molecule unfolds, the pipette is moved away from the optical trap; when it folds the micropipette is moved towards the optical trap. Force is measured at 1 KHz sample rate.
Chapter IV
4. Single molecule studies of ACBP unfolding/refolding pathways

4.1 Introduction
Proteins are subject to mechanical stress in a variety of biological processes [164]. Extracellular matrix proteins, such as fibronectin and tenascin, as well as intracellular cytoskeletal proteins, such as spectrin and actinin, resist mechanical stress to help retain the shape of tissues and cells. Other proteins yield to mechanical perturbation to allow their translocation from one cellular compartment to another. For example, many mitochondrial proteins synthesized in the cytosol must be mechanically unfolded for translocation into mitochondria [165]. Mechanosensors respond to force with subtle structural deformations that mediate the transduction of mechanical stimuli into cellular processes [166-168]. Understanding the basic principles that govern the response of proteins to force will provide insight about the mechanisms by which fundamental biological processes in the cell are influenced and regulated by direct mechanical interactions.

Recent advances in single molecule manipulation techniques, such as atomic force microscopy (AFM) and optical tweezers, have opened new prospective for the study of the behavior of individual proteins under mechanical stress. Using these techniques it is now possible to apply mechanical force along a well-defined molecular axis and characterize the sequence of events that lead to the loss or gain of a protein’s secondary and tertiary structure. To date, the large majority of mechanical manipulation studies have been carried out using AFM. In these experiments long polymeric proteins -either naturally occurring [169] or biochemically synthesized [170], are stretched between a solid substrate and a silicon nitride tip to induce and characterize the mechanical unfolding of the individual monomeric units. These studies have provided a wealth of new information about the mechanical properties of different proteins, but they have been mostly limited to the characterization of the high unfolding behavior of proteins. The high spring constant of AFM probes has made it very difficult to observe low-force unfolding and refolding events. These experimental limitations have recently been overcome through the development of methods to manipulate individual proteins in the low force regime of optical tweezers [62]. Using DNA molecules as molecular handles is now possible to manipulate single proteins with optically trapped beads and monitor in real time refolding events as well fluctuations between different molecular conformations. The power of this recently developed experimental approach to study protein folding has been already proved on different molecules by different authors.
In this work we use a dual-beam optical tweezers to study the behavior of individual bovine Acyl-coenzyme A binding protein (ACBP) under tension. ACBP is a very simple and highly conserved 4-helix bundle protein made of 86 amino acids. It is an excellent molecule to study protein folding as it contains no disulfide bonds or prosthetic groups, and its three-dimensional structure has been solved for different variants. Moreover, the wealth of thermodynamic and kinetic information already available for this protein through bulk experiments can be used to interpret and reinforce single molecule data.

4.2 Theory
Before illustrating the results of our studies, I will describe the theoretical models used to interpret and analyzed the experimental data.

4.2.1 Entropic elasticity
A polymer in solution bends and curves locally as a result of thermal fluctuations and assumes a random coiled structure that maximizes its entropy. If the polymer is extended, an opposing force is generated as a result of the reduction in entropy. This purely entropic elastic behavior is called entropic elasticity. The entropic elasticity of polymers is usually described by the inextensible worm-like chain (WLC) model [171], which treats the polymer as a flexible rod of length $L$ that curves smoothly as a result of thermal fluctuations. The directional correlation between two segments in the polymer decreases exponentially with their separation $s$ according to $e^{-s/P}$, where the decay length $P$ is the persistence length of the polymer. The stiffer is the polymer, the larger is $P$. The exact force $F$ required to extend a polymer of contour length $L$ must be obtained numerically, but an useful approximation is given by the interpolation formula:

$$\frac{FP}{k_BT} = \frac{1}{4\left(1 - \frac{x}{L}\right)^2} + \frac{x}{L} - \frac{1}{4}$$

(1)

where $k_BT$ is the thermal energy and $x$ is the end-to-end distance of the polymer.

4.2.2 Effect of force on the thermodynamics of single molecule reactions
The effect of force on the thermodynamics of a single molecule reaction can be determined using standard thermodynamic theory with force and length being the one-dimensional
equivalents of the fundamental thermodynamics parameters of pressure and volume [172]. The free energy change to unfold a single molecule at zero force is

$$\Delta G_{H_P}^0 = -k_B T \ln K_{eq}$$

(2)

where $K_{eq}(F)$ is the equilibrium constant for the folding/unfolding process.

At constant temperature and pressure, the reversible work to unfold a molecule is:

$$W_{rev}(F) = \Delta G_{H_P}^0 + k_B T \ln K_{eq}(F) + \Delta G(F)_{stetching} = \int_0^{\Delta x(F)} \langle F(x') \rangle \, dx'$$

(3)

where $\Delta G(F)_{stetching}$ is the work done to stretch the unfolded state. When the force is such that the molecule has equal probability of being either in the folded or unfolded state ($F_{1/2}$), $K_{eq}(F) = 1$ and $k_B T \ln K_{eq}(F) = 0$. As a consequence:

$$W_{rev}(F_{1/2}) = \Delta G_{H_P}^0 + \Delta G(F_{1/2})_{stetching} = \int_0^{\Delta x(F_{1/2})} \langle F(x') \rangle \, dx' \approx F_{1/2} \Delta x(F_{1/2})$$

(4)

Thus, at force equal $F_{1/2}$, the free energy of unfolding of a molecule can be calculated as the area under the unfolding or refolding transitions observed in force vs extension plots. This free energy however can not be compared with that obtained at zero force with bulk methods, unless the stretching free energy is calculated and subtracted. With good approximation, $\Delta G(F_{1/2})_{stetching}$ can be calculated as the area under the WLC force-extension curve integrated from zero to the extension of the unfolded molecule at $F_{1/2}$. This latter extension can be determined by increasing the length of the unfolding rip at $F_{1/2}$ by the length of the folded molecule.

### 4.2.3 Effect of force on the kinetics of single molecule reactions

If we consider a molecule in solution whose unfolding and refolding is a two-state process, then, according to the transition state theory, the rate of unfolding ($k_u^0$) and refolding ($k_f^0$) of the molecule at force equal zero are given by Eq. (5) and (6), respectively:

$$k_u^0 = A \exp (-\Delta G_u / k_B T)$$

(5)
where $A$ is the natural frequency of oscillation, $\Delta G_u$ and $\Delta G_f$ are the activation energy for unfolding and refolding respectively, and $k_B T$ is the thermal energy.

When tension is applied to the molecule, to a first approximation, we can assume that the applied force lowers the free energy of the reaction by an amount linearly dependent on the position along the coordinate [164]. This is equivalent to say that the force tilts the free-energy profile of the unfolding/refolding reaction with a pivot point at $X=0$, without changing the shape of the energy landscape (Figure 4.1).

\[ k_f^0 = A \exp \left( -\frac{\Delta G_f}{k_B T} \right) \]

\[ k_u^0 = A \exp \left( -\frac{\Delta G_u}{k_B T} \right) \]

\[ k_u(F) = k_u^0 \exp \left( \frac{-FX_u}{k_B T} \right) \]

\[ k_f(F) = k_f^0 \exp \left( \frac{FX_f}{k_B T} \right) \]

\[ k_u(F) = k_u^0 \exp \left( \frac{-FX_u}{k_B T} \right) \]

\[ k_f(F) = k_f^0 \exp \left( \frac{FX_f}{k_B T} \right) \]

**Figure 4.1: Effect of force on the energy landscape of a molecule that unfolds and refolds in a two-state manner.**

As a result, the unfolding activation energy $\Delta G_u$ is lowered by an amount equal to $F \Delta X_u$, where $\Delta X_u$ is the distance between the native state and the transition state, while $\Delta G_f$ is increased by an amount equals to $F \Delta X_f$, where $\Delta X_f$ is the distance between the unfolded state...
and the transition state. The rates of unfolding and refolding as a function of the applied 
force are then as in Eq. (7) and (8),

\[ k_u(F) = A \exp \left\{ -\left( \Delta G_u - F \Delta x_u \right)/k_B T \right\} = k_u^0 \exp \left( F \Delta x_u/k_B T \right) \]  

(7)

\[ k_f(F) = A \exp \left\{ -\left( \Delta G_f + F \Delta x_f \right)/k_B T \right\} = k_f^0 \exp \left(-F \Delta x_f/k_B T \right) \]  

(8)

Notice that in optical tweezers experiments the measured \( k^0 \) are the rates with which the 
etire system (beads, handles, and protein) fluctuates from one extension to another. The 
measured \( k^0 \) can be seen as the product of the true \( k^0 \) of the protein and a machine constant 
\( k_m \). Thus rates measured in these experiments can not be compared with rates obtained 
through bulk experiments.

### 4.2.4 Probability of (un)folding

If unfolding is a 1\textsuperscript{st} order kinetic reaction, and the refolding rate is negligible, the time 
dependence of the probability that the reaction has not occurred is [172]:

\[ \frac{dP_f(t)}{dt} = -k_u(t) P_f(t) \]  

(9)

If a molecule is subject to a force that varies linearly with time \( F=at \), where \( a \) is the loading 
rate (pN/sec), then we can change variable from \( t \) to \( F \) in Eq. (9) to obtain:

\[ \frac{dP_f(F)}{dF} = -\frac{k_u(F)}{a} P_f(F) \]  

(10)

Considering Eq. (7) and integrating from force zero to \( F \),

\[ \int \frac{dP_f(F)}{P_f(F)} = -\frac{k_u^0}{a} \int \exp(F \Delta x_u/k_B T) dF \]  

(11)

We obtain,

\[ \ln\{P_f(F)\} = \frac{k_u^0 k_B T}{a \Delta x_u} \left( 1 - \exp(F \Delta x_u/k_B T) \right) \approx -\frac{k_u^0 k_B T}{a \Delta x_u} \exp(F \Delta x_u/k_B T) \]  

(12)
The relevant range of Eq. (12) is when the probability of the reaction is significant; this means that the exponential term is large compared to 1. We thus obtain for the force dependence of the reaction

\[
\ln \{ P_f (F) \} = - \frac{k_u^0 k_B T}{\Delta x_u} \exp(F \Delta x_u / k_B T)
\]  

(13)

Equation (13) can be linearised and used to fit unfolding force distributions in order to estimate \( k_u^0 \) and \( \Delta x_u \).

\[
\ln \{ a \ln (1 / P_f (F)) \} = \ln \frac{k_u^0 k_B T}{x_u} + (\Delta x_u / k_B T) F
\]  

(14)

The slope of a plot of Eq. (14) gives the distance to the transition state; the intercept plus slope gives \( k_u^0 \)

Similar consideration applies for refolding

\[
\ln \{ -a \ln (1 / P_u (F)) \} = \ln \frac{k_f^0 k_B T}{x_f} + (\Delta x_f / k_B T) F
\]  

(15)

### 4.3 Force-ramp experiments

In these experiments, individual ACBP molecules were stretched and relaxed multiple times by moving the micropipette relative to the optical trap. The micropipette was moved at constant speeds (nm/sec) to generate almost constant loading/relaxation rates of pulling (pN/sec) [173].

During the experiment, the force applied on the protein was determined by measuring the change in momentum flux of the light beams leaving the trap, while the extension of the molecule was determined using a “light lever” system [1]. Force vs extension plots for ACBP are shown in Figure 4.2. Sudden changes in the extension of the molecule (transitions) during both stretching and relaxation were observed, corresponding to the unfolding and refolding of the molecule. These transitions were not observed during control experiments with DNA handles alone (data not shown). ACBP unfolding occurs at \( \sim 12 \) pN, while refolding occurs at \( \sim 4 \) pN.
Figure 4.2: Mechanical manipulation of a single ACBP molecule by optical tweezers. A) Schematic representation of the experimental strategy. A single protein is manipulated between two polystyrene beads by means of ~500 bp DNA molecules. One bead is held in place at the end of a pipette by suction, while the other is held in an optical trap. During the experiment, the protein is stretched and relaxed by moving the pipette relative to the laser trap by means of a piezoelectric actuator and the forces applied on the molecule are determined by measuring the change in momentum flux of the light beams leaving the trap (16). B) Force vs extension cycle obtained by stretching (red) and relaxing (blue) an ACBP molecule with optical tweezers. The stretching trace shows a discontinuity (rip) at about 12 pN that corresponds to the mechanical denaturation of the molecule. In fact, when the protein unfolds under tension its end-to-end distance suddenly increases as the molecule goes from its compact native state (N) to its extended unfolded state (U), (N to U transition). On the other hand, upon relaxation of the force ACBP refolds into N at about 4 pN giving rise to a rip in the relaxation trace that restores the original molecular extension (U to N transition). C) Multiple force vs extension cycles overlaid.
The changes in contour length (ΔCl) of the protein associated with the unfolding and refolding events were estimated by fitting the force-extension traces with the worm-like chain model [171], Figure 4.3.

Because ACBP is stretched from position 1 and 86, and the distance between these two amino acids in the folded structure is 2.3 nm, the full unfolding or refolding of the molecule should produce a contour length change of \((85 \times 0.36 \text{ nm}) - 2.3 \text{ nm} = 28.3 \text{ nm}\). WLC fitting yielded: 29.13 \(\pm\) 1.56 nm \((n=91)\) for unfolding and 28.99 \(\pm\) 1.57 nm \((n=56)\) for refolding. Both contour length changes are consistent with a full unfolding and refolding of the protein. These results suggest that the molecule unfolds and refolds in a two-state manner. In fact, intermediate states between N and U were not detectable even in force-extension traces collected at 1000 Hz sampling rate, (data not shown).

For a two state system in which \(k(F)\) depends exponentially on force (Eq. 7 and 8), force distributions can be used to calculate the position of the transition state along the pulling axis and the rate coefficient at zero force using Eq. 14 and 15. The fractions of the unfolded (U) and folded (N) molecules at various forces can be calculated by integrating the histograms of the force distributions over the corresponding range of forces. Figure 4.4 shows plots of \(\ln\{\ln\{1/N\}\}\) and \(\ln\{-\ln\{1/U\}\}\) as a function force.
Figure 4.4 Panels A) and B) show distributions of unfolding and refolding events observed at a loading rate of 8.5 pN/sec and 15 pN/sec, respectively. Panel C) shows a plot of $\ln\{\ln\{1/N\}\}$ and $\ln\{-\ln\{1/U\}\}$ vs force. $N$ and $U$ are the folded and unfolded fractions respectively. Red solid circles and crosses represent unfolding data collected at 8.5 and 15 pN/sec, respectively. Blue solid circles and crosses represent refolding data collected at 8.5 and 15 pN/sec, respectively. Fit of the unfolding data with Eq. 14 yielded: $k_u0 = 3.47 \times 10^{-5} \pm 8.77 \times 10^{-6}$ sec$^{-1}$, $x_u = 5.31 \pm 0.13$ nm. Fit of the refolding data with Eq. 15 yielded: $k_f0 = 3.75 \times 10^3 \pm 5.84 \times 10^2$ sec$^{-1}$, $x_f = 6.73 \pm 0.12$ nm. Using these best fit values and Eq. 7 and 8 we calculated a
Unfolding and refolding data acquired at different loading rates appear to overlap. Fits of these data with Eq. 14 and 15 yielded: \( k_u = 3.47 \times 10^{-5} \pm 8.77 \times 10^{-6} \) sec\(^{-1}\), \( x_u = 5.31 \pm 0.13 \) nm, and \( k_f = 3.75 \times 10^{3} \pm 5.84 \times 10^{2} \) sec\(^{-1}\), \( x_f = 6.73 \pm 0.12 \) nm. By plugging these values in equations 7 and 8 we determined the force (F/2) at which the unfolding and refolding rates are equal and thus \( K_{eq} (F) = 1 \). F/2 is 6.32 pN. As explained above, at F/2, the change in free energy of the unfolding or refolding reaction is equal to the reversible work \( F \Delta X \), where \( \Delta X \) is the change in end-to-end distance of ACBP upon unfolding and refolding at F/2. Using a \( \Delta X \) of 12.3 +/- 0.4 nm (Figure 4.5), we calculated the reversible work to be 11.15 +/- 0.6 Kcal/mol.

\[ \Delta G_{\text{stretch}} \] as the area under the worm like chain curve from zero to F/2 force. We obtained a \( \Delta G_{\text{stretch}} \) of 5.6 +/- 0.5 Kcal/mol. Thus \( \Delta G_0 \) for ACBP in our experiments appears to be 5.6 +/- 1 Kcal/mol. This value compares well with that measured in bulk experiments, \( \Delta G_0 = 7.06 +/- 1.36 \) Kcal/mol, [174].

**Figure 4.5: Force constant measurement.** In black is shown an extension vs time trace of ACBP at 6.3 pN of force. The protein hops between its unfolded and folded state with a change in extension of 12.3 +/- 0.4 nm. In red is shown a time trace of force under feedback control. The force is kept constant at 6.3 pN.

To estimate the standard free energy at zero force (\( \Delta G_0 \)), we calculated \( \Delta G_{\text{stretch}} \) as the area under the worm like chain curve from zero to F/2 force. We obtained a \( \Delta G_{\text{stretch}} \) of 5.6 +/- 0.5 Kcal/mol. Thus \( \Delta G_0 \) for ACBP in our experiments appears to be 5.6 +/- 1 Kcal/mol. This value compares well with that measured in bulk experiments, \( \Delta G_0 = 7.06 +/- 1.36 \) Kcal/mol, [174].
4.4 Force-jump experiments

To observe unfolding and refolding events at equilibrium, constant force experiments can be performed in which the extension of the molecule is monitored over time while the applied force is held constant at a preset value by a feedback mechanism. In these experiments, rate coefficients can be obtained directly from the lifetimes of the folded and unfolded states, and free energies can be calculated from the ratio of the kinetics coefficients. This experimental approach however is effective only when the rate of the forward and reverse reaction is high enough to allow the acquisition of a large number of events in a relative short amount of time. Around 6.5 pN, ACBP unfolds and refolds at equilibrium. However, the interconversion rate between the folded and unfolded states is so low that the acquisition of a statistically significant number of events would require hours of recording. Taking data in a reliable manner for such long period of time requires an instrumental stability that our setup does not have. To overcome this problem we performed force-jump experiments.

In force-jump experiments the force is increased (jumped) or decreased (dropped) quickly to a preset force value and kept constant with a feedback mechanism until an unfolding or refolding event is observed. These experiments allow the direct measurement of rate constants in force ranges where the probability of observing either an unfolding or refolding event is high. A typical force-jump experiment with ACBP is shown in Figure 4.6. At the beginning, the force is 6 pN, and the protein is folded. Then F is quickly raised and kept constant at 8.7 pN until the protein unfolds, as indicated by a sudden increase in its end-to-end distance. The force is then suddenly dropped to 6 pN and held constant until a decrease in molecular extension signals the refolding transition. The force-jump/force-drop cycle can be repeated multiple times until a large number of events have been observed. At each cycle, the time that the molecule takes to unfold ($\tau_u$) or refold ($\tau_f$) can be directly measured. Fig. 4.6B and C show the dwell time distributions of ACBP at 8.7 and 6 pN, respectively. These distributions can be well fitted by single exponentials, proving the first order nature of the unfolding/refolding transitions of this protein. Distributions of $\tau_u$ and $\tau_f$ were measured at different forces and unfolding/refolding rates were determined through single exponential fits.
Figure 4.6: A) Two force-jump cycles. In black is shown ACBP extension vs time. Upon unfolding or refolding the extension of the molecule suddenly increases or decreases, respectively. In red is shown the force applied on the molecule vs time. There are some transient deviations from the preset force values of 6 and 8.7 pN that coincide with the unfolding/refolding events. These force changes reflect the lag of the feedback mechanism. Panels B) and C) show dwell time histograms of the folded and unfolded states measured at 8.7 and 6 pN respectively. Single exponential fits to the data (solid lines) yielded a ku of 0.37.
Figure 4.7 show the graph of lnk vs F for ACBP obtained through force-jump experiments.

Figure 4.7: Plots of the logarithm of the rate constants vs force for unfolding (red dots) and refolding (blue dots). Fit of the unfolding data with the logarithmic form of Eq. 7 yielded: $k_{u0} = 7.5 \times 10^{-6} \pm 9.58 \times 10^{-6}$ sec$^{-1}$, $x_u = 5.47 \pm 0.64$ nm. Fit of the refolding data with the logarithmic form of Eq. 8 yielded: $k_{f0} = 1.71 \times 10^3 \pm 5.93 \times 10^2$ sec$^{-1}$, $x_f = 6.48 \pm 0.28$ nm. These data were fit with logarithmic forms of Eq. 14 and 15 to estimate the position of the transition states along the pulling axis and the rate coefficients at zero force. This analysis yielded: $k_{u0} = 7.5 \pm 9.58 \times 10^{-6}$ E-6 sec$^{-1}$, $x_u = 5.47 \pm 0.64$ nm, and $k_{f0} = 1.71 \pm 5.93 \times 10^2$ E+2 sec$^{-1}$, $x_f = 6.48 \pm 0.28$ nm. The crossing point of the fitting lines in Figure 4.7 marks the force ($F_{1/2}$) at which $k_u$ and $k_f$ are equal, and thus $K_{eq} = 1$. $F_{1/2}$ was estimated to be 6.63 pN. Using this $F_{1/2}$ value and a $\Delta X$ of 12.3 $\pm$ 0.4 nm, we estimated the reversible work to unfold ACBP to be 11.7 $\pm$ 0.5 Kcal/mol. In this case $\Delta G_{stretch}$ was calculated to be 5.7 $\pm$ 0.5 Kcal/mol and thus $\Delta G_0$ to be 6 $\pm$ 1 Kcal/mol. This change in free energy is similar to the one measured in bulk [174]. Notice that the sum of the distances to the transition state measured with force-ramp and force-jump experiments (12.04 $\pm$ 0.25 nm and 11.95 $\pm$ 0.92 nm respectively) is consistent with the change in end-to-end distance of the molecule measured at 6.3 pN ($\Delta X = 12.3 \pm 0.4$ nm).
4.5 Comparison of force-ramp and force-jump results

Table 4.1 summarizes the kinetics and thermodynamics value obtained with force-ramp and force-jump methods.

<table>
<thead>
<tr>
<th></th>
<th>$k_u''$ (sec$^{-1}$)</th>
<th>$x_u$ (nm)</th>
<th>$k_f''$ (sec$^{-1}$)</th>
<th>$x_f$ (nm)</th>
<th>$F_{1/2}$ (pN)</th>
<th>$\Delta G_0$ (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force-</td>
<td>3.47 +/- 0.877 E-5</td>
<td>5.31 +/- 0.13</td>
<td>3.75 +/- 0.58 E+3</td>
<td>6.73 +/- 0.12</td>
<td>6.32</td>
<td>5.6 +/- 1</td>
</tr>
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<td>ramp</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Force-</td>
<td>7.5 +/- 9.58 E-6</td>
<td>5.47 +/- 0.64</td>
<td>1.71 +/- 0.59 E+3</td>
<td>6.48 +/- 0.28</td>
<td>6.63</td>
<td>5.7 +/- 1</td>
</tr>
<tr>
<td>jump</td>
<td></td>
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</tbody>
</table>

Table 4.1: Kinetics and thermodynamics data obtained with force-ramp and force-jump methods.

With Eq. 7 and 8, using the best fit values of figure 4.4C, we calculated $\ln k$ vs force plots for force-ramp measurements and showed them together with those obtained with force-jump experiments, Figure 4.8.

Figure 4.8: Comparison of rates measured with different methods. Red and blue dots represent data obtained with force-jump experiments. Red and blue triangles show rates calculated with Eq. 7 and 8, using $k$ and $x$ values obtained by fitting unfolding and refolding force distributions (Fig. 4.4C).
4.6 Molecular Dynamics (MD) simulations
MD simulations were performed by Dr. G. Tiana and C. Camilloni and the results of these studies are shown here for completeness.

The possibility of interpreting the data obtained by optical tweezers at an atomic-scale resolution by means of molecular dynamics simulations is hampered by the fact that at forces of few pN proteins unfold in times that are not reachable by standard computational techniques. As a matter of fact, so far a theoretical description of mechanical unfolding was carried out only either with very simple models [175], which lack of a real atomic detail, or using forces which are larger by orders of magnitude than those used in tweezer experiments [176].

This problem can be partially solved using ratcheted molecular dynamics simulations, which are able to extract the sequence of conformational changes between given points of the conformational space, provided that a good reaction coordinate is given [177, 178]. Ratcheted simulations employ a standard explicit-solvent force field (Amber in the present case), to which we add a soft biasing potential which depends on the value \( y \) of the reaction coordinate in the harmonic form

\[
U_{rat}(t) = \frac{1}{2} k (\rho(t) - min_{t'<t} \rho(t'))^2
\]  

(16)

Where

\[
\rho(t) = (y(t) - y_{target})^2
\]  

(17)

\( k \) is the harmonic constant, \( y \) is the reaction coordinate (which is the distance between cysteines 1 and 86 in the unfolding simulations), and \( y_{target} \) is the wished ending point of the simulation (which is infinity for the unfolding simulations). If the value of \( k \) is small enough, the trajectories obtained with ratcheted molecular dynamics are a good approximation of the actual trajectories. The price to be paid is that the timing of the trajectories and the equilibrium properties are lost. This technique has proven successful in studying the sequence of events along the thermal unfolding of fibronectin and along the spontaneous folding of protein G, CI2 and ACBP [177]. Using a full atomic description of ACBP in explicit water, we have generated different sets of six unfolding trajectories each, at various intensities of the (constant) applied force.
A clear picture of the order of contact formation/disruption can be obtain from Figure 4.9, which displays snapshots of the simulated trajectories for selected distances $d$ between the ends of the protein.

![Figure 4.9: Snapshots of the unfolding trajectories at $k=12$ pN selected at different values of the distance $d$ between the two cysteines. Above the snapshots associated to each value of $d$ is indicated their structural variability.](image)

Unfolding at a force of 12 pN takes place in a rather homogeneous way, the average RMSD for conformations at a given value of $d$ ranging $0.21$ nm to $1.15$ nm. Unfolding starts with the disruption of the tertiary contacts between h1 and the rest of the protein and subsequently of the secondary contacts in h1. Then h4 starts losing both its secondary and tertiary contacts. The structure involving h2 and h3 is the last to be lost before complete unfolding. A more detailed analysis can be obtained by the order of native-contact disruption displayed at residue level in Figure 4.10.
The very first native contacts to be lost are at both ends of h1, and the non-local contacts 21-53, 28-73, 11-86, 14-86, 2-70, 15-86. Some small rearrangements takes place also in h2 (cf. S20-25, 33-39), while h3 remains intact. Among the last native contacts to be disrupted are 39-58, 29-61, 25-57, and the central secondary contacts in h2 and h3.

Another piece of information that can be extracted from the simulations is about the transition states. Even if the transition state is a feature associated with the free energy of the system, which in principle cannot be extracted from ratcheted simulations, we can obtain the structure of the transition state with an indirect method. The idea is the following. When crossing a free-energy barrier, the system goes from a situation where it climbs the free energy barrier running against the molecular forces, to a situation where the molecular forces make it descend towards the following minimum. In the former case, the ratchet plays an important role, and the value of rho is in average larger than its minimum (i.e., the value of $U_{rat}$ is in average large). In the latter case, rho is close to its minimum and $U_{rat}$ is approximately zero. The point where the system changes its behaviour from the former to the latter is the transition state Figure 4.11.
In the unfolding transition state, the distance $d$ between the ends of the protein is $5.78 \pm 0.22$ nm, to be compared with the value 5.46 nm obtained in the unfolding experiments. The structure of the transition state is quite similar in all trajectories, the average RMSD being 0.44 nm. In such a transition state the secondary contacts of h4 and the structure h2-h3 are still formed. The transition state along has also been studied at different values of the applied force both for unfolding and folding simulations, and the resulting values of $d$ are plotted in Figure 4.12.

Considering the error bars displayed in the figure, the position of the transition state results rather independent on the applied force, except at very low values of the force. This justifies the assumption made in the treatment of the experimental data.
Figure 4.12: The average distance between the cyteines in the transition state calculated from unfolding (black symbols) and folding (red symbols) ratcheted MD trajectories at different values of the force $k$. The error bars indicate the standard deviation.

4.7 Pulling ACBP along different molecular axis
I have also started experiments to study the anisotropy of the energy landscape of ACBP. To this end we generated ACBP mutants bearing cysteine residues at positions: 36-86, 46-86 and 17-86. We attached handles at these positions and pulled the molecule along the axes shown in figure 4.13.

Figure 4.13: ACBP NMR structure. Red dots mark the positions were cysteines were engineered on the surface of the protein. Black arrows show the four different pulling axes used to manipulate the ACBP.
Figure 4.14 displays the force extension cycles obtained with these mutants.

![ACBP 1-86](image1)

![ACBP 17-86](image2)

![ACBP 36-86](image3)

![ACBP 46-86](image4)

**Figure 4.14:** Force vs extension cycles of ACBP pulled along different molecular axes.

In all cases, unfolding and refolding of ACBP give rise to clear transitions in the force vs extension traces. Similarly to what we have done with 1-86 ACBP, we have started analyzing the denaturation of these variants using both force distributions and force-jump experiments. Figure 4.15 show the results of force-jump experiments.

![ln k vs Force](image5)

**Figure 4.15:** Force-jump experiments
Table 4.2, 4.3 and 4.4 summarize the kinetics data collected so far with these three ACBP variants.

<table>
<thead>
<tr>
<th></th>
<th>$k_u^u$ (sec$^{-1}$)</th>
<th>$x_u$ (nm)</th>
<th>$k_f^u$ (sec$^{-1}$)</th>
<th>$x_f$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force-ramp</td>
<td>3.23 +/- 0.5 E-4</td>
<td>4.17 +/- 0.13</td>
<td>2.55 +/- 0.58 E+3</td>
<td>6.87 +/- 0.77</td>
</tr>
<tr>
<td>Force-jump</td>
<td>2.93 +/- 0.6 E-4</td>
<td>3.24 +/- 0.64</td>
<td>1.34 +/- 0.3 E+4</td>
<td>7.86 +/- 0.32</td>
</tr>
</tbody>
</table>

*Table 4.2: Kinetics data for 17-86*

<table>
<thead>
<tr>
<th></th>
<th>$k_u^u$ (sec$^{-1}$)</th>
<th>$x_u$ (nm)</th>
<th>$k_f^u$ (sec$^{-1}$)</th>
<th>$x_f$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force-ramp</td>
<td>1.67 +/- 0.4 E-5</td>
<td>1.8 +/- 0.15</td>
<td>1.95 +/- 0.58 E+4</td>
<td>5.4 +/- 0.54</td>
</tr>
<tr>
<td>Force-jump</td>
<td>9.9 +/- 2.58 E-4</td>
<td>1.98 +/- 0.32</td>
<td>5.9 +/- 0.2 E+4</td>
<td>5.61 +/- 0.48</td>
</tr>
</tbody>
</table>

*Table 4.3: Kinetics data for 36-86*

<table>
<thead>
<tr>
<th></th>
<th>$k_u^u$ (sec$^{-1}$)</th>
<th>$x_u$ (nm)</th>
<th>$k_f^u$ (sec$^{-1}$)</th>
<th>$x_f$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force-ramp</td>
<td>5.27 +/- 1.3 E-5</td>
<td>2.69 +/- 0.57</td>
<td>4.15 +/- 1.2 E+4</td>
<td>3.4 +/- 0.32</td>
</tr>
<tr>
<td>Force-jump</td>
<td>2.86 +/- 0.58 E-4</td>
<td>2.04 +/- 0.44</td>
<td>1.76 +/- 0.59 E+5</td>
<td>3.71 +/- 0.52</td>
</tr>
</tbody>
</table>

*Table 4.4: Kinetics data for 46-86*

Notice that the sums of the distances to the transition state for all mutants are consistent with what expected from the WLC model.
Chapter V
5. Conclusions
In this thesis I have reported the results of single molecule studies of the unfolding/refolding trajectories of the protein ACBP. These experiments were carried out using a custom-built optical tweezers setup that operates through direct measurements of light momentum. I devoted a significant part of my doctorate studies to the development of this instrument. Specifically I contributed to: 1) the development of programs in LabWindows/CVI to perform force-ramp and force-jump experiments, 2) the development of data acquisition programs, 3) the alignment of optics, and 4) the reduction of mechanical and optical noise. I also developed programs in LabView to analyze optical tweezers data.

In collaboration with Pétur Orri Heiðarsson from the Department of Biology, University of Copenhagen, Denmark, I prepared protein-DNA chimeras for use in single molecule optical tweezers experiments and studied the unfolding/refolding processes of ACBP under mechanical force. Most of the data were collected with the 1-86 ACBP variant in which one cysteine was introduced at the N-terminal and one cysteine at the C-terminal by site-directed mutagenesis. This protein was stretched and relaxed multiple times in force ramp experiments to obtain distributions of unfolding/refolding transitions at different loading rates. The behavior of ACBP under mechanical stress was also analyzed through force-jump experiments in which the force is jumped or dropped quickly to preset values and kept constant until an unfolding or refolding event is observed. These experiments allow the direct measurement of rate constants in force ranges where the probability of observing either an unfolding or refolding event is high. The resulting dwell time distributions were analyzed to obtain unfolding and refolding rate constants as a function of force. The atomistic details of the mechanical unraveling of ACBP have been studied by MD simulations by Dr. G. Tiana and Dr. C. Camilloni.

The results of these studies show that, under tension, ACBP unfolds at ~ 10 pN and refolds at ~ 4 pN. The molecular structure of this protein displays unusual mechanical properties as it is able to undergo significant deformation (~ 5 nm in length) before committing to unfolding. To our knowledge, this is the most compliant protein structure so far observed in single molecule manipulation studies. Under our experimental conditions ACBP unfolds and refolds in a two-state manner through memory less Markovian processes. No unfolding or refolding intermediate states were observed in our force-ramp and force-jump experiments. MD simulations of the mechanical denaturation of ACBP reveal a sequence of events in which unfolding starts with the disruption of the tertiary contacts.
between h1 and the rest of the protein, followed by the complete unfolding of h1. Then h4 starts losing both its secondary and tertiary contacts. Finally, h2 and h3 unfold. The transition state observed in MD simulations is located at 5.7 nm from the native state, in good agreement with experimental data, and its structure is characterized by a folded nucleus comprising h2 and h3 and by secondary contacts in h4.

During the last six months of my graduate studies, I have also started experiments on different ACBP variants where the force is applied along different pulling axes. These experiments are aimed at investigating the anisotropy of ACBP’s energy landscape. In collaboration with Pétur Orri Heiðarsson we have initiated the characterization of the mechanical unfolding/refolding processes of 17-86, 36-86 and 46-86 ACBP through force-ramp and force-jump experiments.
References


C. Cecconi, et al., pp. Protein-DNA chimeras for single molecule mechanical folding studies with the optical tweezer. (Submitted to Protein Science).


