Published in final edited form as:

Methods. 2010 September ; 52(1): 3–11. doi:10.1016/j.ymeth.2010.04.015.

Nanosecond Time-Resolved Polarization Spectroscopies: Tools for Probing Protein Reaction Mechanisms

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Abstract

Polarization methods, introduced in the 1800's, offered one of the earliest ways to examine protein structure. Since then, many other structure-sensitive probes have been developed, but circular dichroism (CD) remains a powerful technique because of its versatility and the specificity of protein structural information that can be explored. With improvements in time-resolution, from millisecond to picosecond CD measurements, it has proven to be an important tool for studying the mechanism of folding and function in many biomolecules. For example, nanosecond time-resolved CD (TRCD) studies of the sub-microsecond events of reduced cytochrome *c* folding have provided direct experimental evidence of kinetic heterogeneity, which is an inherent property of the diffusional nature of early folding dynamics on the energy landscape. In addition, TRCD has been applied to the study of many biochemical processes, such as ligand rebinding in hemoglobin and myoglobin and signaling state formation in photoactive yellow protein and prototropin 1 LOV2. The basic approach to TRCD has also been extended to include a repertoire of nanosecond polarization spectroscopies: optical rotatory dispersion (ORD), magnetic CD and ORD, and linear dichroism. This article will discuss the details of the polarization methods used in this laboratory, as well as the coupling of timeresolved ORD with the temperature-jump trigger so that protein folding can be studied in a larger number of proteins.

Keywords

Time-resolved; Circular dichroism; Temperature-jump; Optical rotatory dispersion; Magnetic circular dichroism; Polarization spectroscopy

1. INTRODUCTION

Our current understanding of protein functions such as ligand binding and signal transduction and of protein and peptide folding mechanisms has been aided significantly by the use of structure-sensitive time-resolved spectroscopies. Advances in optical spectroscopies, such as absorption, polarization (circular dichroism, CD; linear dichroism, LD; magnetic circular dichroism MCD), vibrational (infrared, IR; resonance Raman, RR) and fluorescence are providing increasingly structure-specific time-resolved information about proteins[1–3]. At

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the same time, progress in traditional structural probes such as X-ray crystallography and electron spin and nuclear magnetic resonance spectroscopies provide atomic level structural information with increasing time resolution [4,5]. With this rich source of methods available to study proteins, excitement arises not only from the unprecedented information that each probe can offer, but in the overall story that the probes together will tell about folding and functional reactions in biochemical systems. That is, without considering information from different structural probes the picture could be incomplete and possibly inaccurate.

Ideally, conclusions about the folding or functional dynamics of a particular protein are best drawn from the results of many different probes. This, however, is not always possible because of experimental considerations. For instance, pH jump methods [6,7] present a challenge for far-UV time-resolved CD (TRCD) methods if the proton source absorbs too highly in the far-UV region. The success of time-resolved infrared (TRIR) protein folding studies that use high concentrations of denaturant has been limited because guanidine hydrochloride (GuHCl) and urea have strong IR bands that mask the weaker amide I bands of the polypeptide backbone and make solvent subtraction unreliable [8]. Even within a given spectroscopy, such as vibrational (IR vs. FTIR or Raman versus RR and surface-enhanced Raman scattering) or polarization (CD in the electronic versus vibrational spectral regions), changing a parameter can present a probe that provides different types of structure information and may have different experimental limitations. For example, the use of Raman spectroscopy is not optimal for protein molecules because the large number of vibrational modes complicates the spectrum. However, with TR³ spectroscopy, by tuning the excitation laser to coincide with an electronic transition of a particular chromophore the Raman scattering intensity due to the vibrational modes for that transition is substantially enhanced [9]. This feature allows specific parts of a molecule, such as the prosthetic, heme group in hemoglobin to be reflected in the RR spectrum [10]. For protein dynamics, excitation in the visible region does not directly probe protein structure. However, if the excitation wavelength is tuned to the UV and deep-UV regions, vibrational signatures of the amide chromophore and the aromatic amino acids provides information about protein secondary and tertiary structure, respectively [11–13].

Polarization spectroscopy also offers advantages in its versatility and specificity. For example, the structural dynamics of the heme group, in contrast to the protein conformational changes, in cytochrome *c* can be obtained with TRCD measurements in the visible and the far-and near-UV regions, respectively [14]. The power of the CD method is extended with the addition of a magnet to the TRCD configuration [15]. This time-resolved MCD (TRMCD) method is a sensitive probe of structural features that perturb the electronic states of an MCD active chromophore [16]. TRMCD spectroscopy has been applied to the studies of the axial ligation, spin, and oxidation states of the heme iron atom in functional studies of hemoglobin, myoglobin, and cytochrome *c* oxidase [17–20] and in folding studies of cytochrome *c* [15, 21], and more recently has been used to probe the dynamics of the tryptophan residue in hemoglobin [22]. Application of the CD method, which typically probes electronic transitions (also known as ECD), to the vibrational region (VCD) provides more localized sampling of structure. The short-range vibrational coupling and weak dipole moments give VCD the advantage of allowing discrimination of the sheet, helical, and disordered amide band shapes [23]. For protein dynamics, time-resolved VCD data are not currently available. However, successful proof-of-principle experiments with picosecond time resolution have been reported recently [24,25]. The development of synchrotron radiation CD (SRCD) [26] allows lower wavelengths (< 190 nm) of the UV-Vis spectrum to be probed. In that region, the SRCD intensity is orders of magnitude higher than that available from a conventional CD machine, such that the signal-to-noise ratios are greater for measurements on the former. In addition, the CD data measured below 190 nm has higher information content so that more types of secondary structure can be identified. Clearly, each structure sensitive probe has limitations, but together they modify or support the interpretations offered by each method alone.

2. Circular Dichroism, A Short Time-resolved History

2.1. Nanosecond and Millisecond Circular Dichroism

The development of chiroptical methods in the 1800's offered one of the earliest ways to examine protein structure [27]. Since then, many other structure-sensitive techniques have been developed, but CD remains a powerful technique because by changing the wavelength of detection, by adding a magnet, or by slight modification of the optical components different types of structural information are accessible (see below).

In today's conventional CD spectrophotometers, differential measurement of left and right circularly polarized light (LCP and RCP) is possible with the use of photoelastic modulators (PEM) having resonant frequencies of 1–100 kHz. Although the PEM makes it possible to obtain the high signal-to-noise ratio required to measure the typically small (1 in 10^4) differential absorption of LCP and RCP, it comes at a sacrifice to time resolution. Equilibrium CD measurements have long been used to study the structure of organic molecules, as well as the secondary structure of protein biomolecules, but static measurements could not provide the dynamic details of biochemical processes. Therefore, it was necessary to develop ways to measure dynamic CD signals. In 1974, CD spectroscopy was coupled with stopped-flow and flash photolysis methods [28–30], making it possible to study protein kinetics on time scales as fast as milliseconds and then in 1977, to a temperature-jump (T-jump) trigger for CD measurements as fast as $300\text{ }\mu\text{s}$ [31]. More recently, a continuous flow mixer was coupled with a commercial CD instrument for ca. $400\text{ }\mu\text{s}$ time resolution [32]. Although the greater than 30-fold improvement in time resolution was a significant advancement for CD spectroscopy, the results of studies probing protein folding with millisecond time resolution suggested that many protein conformational events occurred on the nanosecond to early microsecond time scale. Such time resolution was achieved in the 1980s when the TRCD technique advanced to measurements on the nanosecond and picosecond time scales [33,34]. Although initially designed with single wavelength detection [33], nanosecond TRCD spectroscopy in this laboratory has evolved to include multichannel detection [35], other nanosecond polarization methods (TRMCD [16], ultrasensitive time-resolved LD (TRLCD) [36], time-resolved optical rotatory dispersion (ORD) (TRORD) [37] and time-resolved magnetic optical rotatory dispersion (MORD) (TRMORD) [38,39]), extension of the measurements from the visible into the far-UV region [40], faster time resolution ($\sim 10\text{ ns}$) [41], and most recently, it has been coupled with a T-jump trigger [42]. Thus, since the mid-1980s, most submillisecond polarization experiments on biomolecules have been performed with the nanosecond time-resolved methods that were developed in this laboratory.

2.2. Picosecond Circular Dichroism

The picosecond CD system that was reported in 1989 by Xie & Simon was based on the use of high repetition rate picosecond lasers to generate tunable pump and probe pulses [34]. This instrument was based on the absorptive CD approach, with the picosecond probe pulse train rapidly modulated between LCP and RCP by an electro-optic modulator (KD*P longitudinal Pockels cell). The Pockels cell is triggered by high voltage polarity switching to alternate between $+\lambda/4$ wave and $-\lambda/4$ wave retardation. Before the sample was excited, the pump pulses passed through a rotating half-wave plate in order to minimize LD and LB artifacts. Laser-induced changes in CD signal were detected in the output of a PMT that monitors the transmitted probe beam by a lock-in amplifier. This picosecond CD system was used to follow the relaxation dynamics in MbCO after photodissociation of the CO ligand and the primary intermediates in the photocycle of bacterial photosynthetic reaction centers [43,44]. In addition, the picosecond CD apparatus was extended to measurements of MCD and applied to studies of the low to high spin state changes of the heme iron upon photodissociation of the CO ligand in MbCO [45].

Within the last decade, efforts have been made to further develop time-resolved CD methods with picosecond time resolution. Initially using the approach described by Xie and Simon, but applying a rigorous alignment procedure to the Pockels cell to obtain symmetrical circular polarizations [46], Hache and coworkers have since modified their picosecond CD approach to maintain linearly polarized probe light and to measure the probe ellipticity with a Babinet Soleil compensator, set at small retardation, that is positioned between two crossed polarizers after the sample. This single wavelength-detected picosecond CD method has been used to monitor the excited state of ruthenium complexes, the conformational dynamics of photoexcited binaphthol and MbCO [47]

3. Nanosecond Time-resolved Polarization Spectroscopy

3.1. Time-resolved Circular Dichroism

Nanosecond TRCD, TRMCD, TRLD, TRORD, and TRMORD experiments have extended substantially beyond proof-of-principle, with applications to functional studies of phytochrome, carbonmonoxide-bound myoglobin and hemoglobin, cytochrome c oxidase, rhodopsin, photoactive yellow protein (PYP), and the phototropin 1 light-, oxygen-, or voltage regulated (LOV2) domain (vide infra). Far UV TRCD and TRORD and visible TRMCD methods have also been applied to folding studies of cytochrome c, azobenzene cross-linked peptides and RNase A. The method of nanosecond TRCD spectroscopy monitors changes in beam polarization rather than changes in sample absorbance. This approach is based on an early, historical ellipsometric method, where the influence of a circularly dichroic sample on linearly polarized light is determined. The advantage of this null ellipsometric method is that it is the most sensitive approach, although care must be taken to avoid artifacts due to simultaneous detection of circular birefringence (CB) or LD.

The TRCD apparatus shown in Figure 1 uses a modification of the null method, that permits measurement of the sign and magnitude of CD spectra on a nanosecond time scale without spectral distortion from CB. The probe source, a xenon flash lamp, emits initially unpolarized light that is collimated and converted into linearly polarized light when it passes through a polarizer (MgF₂ Rochon prism for the far-UV region; calcite Glan-Taylor polarizer for the near-UV-visible regions) (P1). This quasi-null approach [48] uses a mechanically strained, fused silica plate (SP) to introduce a slight phase retardance, δ (~ 1 – 2°), converting linearly polarized light to elliptically polarized light of high eccentricity. The intensity of the major axis of the elliptical light is about 10^4 more intense than that of the minor axis, which lies with its polarization axis perpendicular to P1. Right elliptically polarized (REP, $+45^\circ$) and left elliptically polarized (LEP, -45°) light are produced by a 180° rotation of the SP. After the SP, which is positioned with the compression (fast) strain axis oriented at $+45^\circ$ or -45° relative to the polarization axis of P1, the elliptically polarized probe beam passes through the sample. Because elliptically polarized light comprises LCP and RCP of different magnitudes, a circularly dichroic sample changes the ellipticity of LEP and REP. Therefore, a circularly dichroic sample either adds to or subtracts from this ellipticity and CB in the sample rotates the axis of the ellipse. The difference between measurements with $\pm \delta$ is proportional to the CD of the sample, and to first order the effect of CB cancels. Changes in the minor axis are detected when the elliptical light passes through a second, analyzing MgF₂ linear polarizer (P2) that is oriented with its polarization axis perpendicular to P1 and subsequently is focused onto the slit of a spectrograph coupled to a multichannel detector. The CD signal is defined by the difference between the intensities of LEP and REP light normalized to their sum, $S = (I_R - I_L)/(I_R + I_L)$.

TRCD has been applied to the functional studies of several proteins including phytochrome in the visible [49] and far-UV [50,51] regions, PYP [52] and LOV2 [53] in the far-UV region, HbCO and MbCO in the visible [33,54,55] and near UV [56] regions, and rhodopsin

photointermediates in the 300–700 nm region [57]. Figure 2a compares the CD spectrum measured 5 ms after photoinitiation of the phytochrome reversion reaction (Pfr (switch-on) to Pr (switch-off)) with that for Pfr. These experiments on phytochrome [50,51] in the 1990's are the first applications of the nanosecond far-UV TRCD method to studies of protein function. Far-UV TRCD has also been used to study folding of cytochrome *c* triggered by ligand photolysis [58] and photoreduction [59]. In Figure 2b, far-UV TRCD spectra are shown at various times after photoreduction of the initially oxidized cytochrome *c* protein. In addition, several inorganic complexes [35,60–62] and transfer RNA [63] have also been investigated with the nanosecond TRCD method.

3.2. Time-resolved Magnetic Circular Dichroism

Natural CD, provides a global picture of biomolecular structure (protein secondary structure, chromophore-protein interactions, relative orientations of dipole-dipole coupled chromophores) derived from the electronic interactions between generally achiral chromophores. In contrast, MCD offers a probe of the protein environment local to a given chromophore through its sensitivity to modulations of chromophore electronic energies by environmental perturbations. This technique has been generally used to study chromophores with large magnetic moments arising from rotational symmetries (aromatics, porphyrins), unpaired spins (metal complexes), or both (hemes). Heme proteins are a classic example of how the complementary techniques of CD and MCD (e.g., the global protein structure is examined by CD and the heme axial ligation and spin state by MCD) can provide a broader understanding of the dynamics of a biomolecule.

The extension of nanosecond TRCD measurements to TRMCD [16] involves the addition of two components, a magnet at the sample and an optical rotator to counteract the Faraday rotation (also known as magnetic optical rotation) of the probe beam polarization that arises from the effect of the magnetic field on the solvent and cell windows. Reliable cancellation is required to avoid interference of this Faraday effect on the measured MCD spectrum. One of the earliest approaches to compensate for the wavelength dependent Faraday rotation of the plane of the probe beam polarization by the solvent and cell in nanosecond TRMCD experiments was the use of a dextrorotatory sucrose solution to rotate the beam polarization back. A sucrose solution works well to back rotate the probe beam polarization, but only into the near-UV region. Towards the far-UV region the solution ORD contributions start to differ from the Faraday dispersion (MORD) of the cell and solvent. A more accurate compensation method that has been implemented in the current nanosecond TRMCD apparatus uses the MORD of a solvent blank with a matched magnet of opposing field direction. The TRMCD signals are measured and calculated in the same manner described above for TRCD.

Since the first studies were reported for the lowest excited triplet state of zinc tetraphenylporphine [16], the TRMCD method has been applied to the functional studies of the photodissociation intermediates of mammalian cytochrome *c* oxidase [17,18], bacterial oxidase cytochrome *ba*₃ [19], myoglobin [39,64], and cytochrome *c*₃ [20]. Two newer directions for TRMCD studies involve studies of the heme-ligand dynamics during folding of cytochrome *c* [15] and near-UV studies of the dynamics of the Trp β37 residue during the earliest steps of ligand rebinding in hemoglobin [22]. An example of structure-rich TRMCD data [15], the results of studies of reduced cytochrome *c* folding initiated by ligand photodissociation, is shown in Figure 2c. Analysis of these data provided strong kinetic evidence that during folding the heme binds two different non-native ligands, histidine and methionine, along parallel pathways that involve different conformational ensembles of the protein. The TRMCD data for studies including and subsequent to reference [15], were calculated from measured TRMORD, as discussed in the next section [21]. The results of the near-UV studies of Trp β37 provide the impetus to further develop TRMCD spectroscopy of

Trp as a conformational probe of protein folding reactions. Because a large number of proteins contain Trp, Trp-TRMCD spectroscopy has the potential for wide applicability and will provide data that is complementary to folding studies that use fluorescence detection.

3.3. Time-resolved Optical Rotatory Dispersion

The historical development of the ORD method predates CD, with the first observation of optical rotation (OR) in the early 1800s by Jean Baptiste Biot and Augustin Fresnel [27]. Subsequent to this report of the rotation of the orientation of linearly polarized light in quartz, the effect was also reported for organic compounds such as turpentine and tartaric acid [65, 66]. During that time, nearly all optical activity was studied by optical rotation. In 1895, the wavelength dependence of optical rotation (ORD) and the unequal absorption of LCP and RCP (CD) in optically active substances were identified (Cotton effect) [66]. Before the advent of spectrophotometers, recording OR progressed from the earliest polarimeter, which comprised sunlight and a sheet of glass, to obtaining OR at specific wavelengths of light (sodium D-line, Hg lines) to measurement of ORD and CD from single wavelength determinations over a wide spectral range in the 1930s.

In the 1950s, a renaissance of ORD was driven by organic chemists who applied this technique to organic compounds such as steroids [67] and biochemists who were interested in protein structure [68]. At about the same time, a quasi-null ORD approach was developed by Keston and Lospalluto [48] and later implemented as the Standard Model D Keston polarimetric attachment in Beckman's DU spectrophotometer. This quasi-null principle is the basis for the design of the TRORD system, introduced in 1995 [37], and is a linear analog of the TRCD configuration described above. In much the same way that for electronic transitions the ordinary dispersion and absorption are intrinsically related, rotatory dispersion (CB) and absorption coefficients (CD) in optical activity are related by the Kramers-Kronig transform [69]. In principle, ORD and CD report the same information (see [59] and [70]), but in practice CD spectroscopy is generally chosen over ORD. This preference is due to the localization of CD to the absorption bands of the chromophore of interest, which makes the measured spectrum more easily interpreted. This is particularly true in the determination of protein secondary structural changes, where an ORD spectrum typically comprises multiple Cotton contributions that make analysis of the structural makeup of the protein spectrum more difficult. There are, however, experimental advantages offered by ORD measurements. Perhaps, the main attraction is that ORD spectra are monitored away from the absorption band, which imparts a signal-to-noise enhancement due to increased probe intensities. A second advantage is the simplicity in the experimental set-up – fewer optical elements reduce the potential for the introduction of artifacts to the TRORD measurement.

The nanosecond ORD system is essentially the same as the TRCD configuration, but without the SP between P1 and P2. Based on the idea that CB from a chiral sample adds to or subtracts from a small reference rotation, β , that is introduced into the probe beam polarization axis, P1 is rotated $\pm \beta$ relative to the cross position of the two polarizers. For nanosecond TRORD studies in the visible regions, β is typically $0.5\text{--}1.5^\circ$, and in far-UV TRORD experiments, β is $1\text{--}3^\circ$; in general $|\text{OR}_{\text{sample}}| \ll \beta \ll 90^\circ$. The ORD signal, the difference between the transmitted intensities measured at $\pm \beta$ normalized by their sum ($S = (I_{+\beta} - I_{-\beta}) / (I_{+\beta} + I_{-\beta})$), is proportional to CB / β .

The nanosecond ORD method has been particularly advantageous for the measurement of protein folding kinetics. Because GuHCl absorbs significantly in the far-UV wavelengths ($< 215\text{ nm}$), only part of the characteristic CD marker for α -helix is accessible in that region when proteins are partly or completely unfolded by denaturant. In contrast, the ORD band for the α -helix structure is red-shifted to 230 nm (see Figure 2d and compare with Figure 2b). Thus, TRORD methods have been used extensively to study the folding kinetics of cytochrome *c*

[70–73], as well as azobenzene cross-linked peptides [74, 75]. Figure 2d shows far-UV TRORD data measured for reduced cytochrome *c* folding in 4 M GuHCl. Comparison of these data and the far-UV TRCD (Figure 2b) and TRORD [70] results measured for reduced cytochrome *c* in 3.3 M GuHCl showed unusual folding kinetic behavior as a function of denaturant. Far-UV TRORD spectroscopy has also been used to study the formation and decay kinetics of the signaling state in PYP [52] and in the phototropin 1 LOV2 domain [53] under near-physiological conditions. The far-UV TRCD/TRORD apparatus has also been coupled to the T-jump trigger for a more general approach to initiating protein folding. However, because of the greater sensitivity of the CD method to artifacts, especially those that are temperature-induced, T-jump initiated reactions are generally probed by TRORD.

As with TRCD and TRMCD, TRMORD is implemented by the addition of a magnet in the TRORD apparatus. Although MCD spectra are easier to interpret than MORD spectra, the signal-to-noise advantage makes MORD an attractive method [38]. In addition, because MCD and MORD are Kramers-Kronig transform mates, in practice, MORD measurements are converted to MCD spectra for analysis. The MORD technique has been applied to the study of photolyzed myoglobin-CO kinetics [39,64] and to the ligand dynamics during folding of wild type horse heart and tuna heart cytochrome *c* and two horse heart cytochrome *c* variants [21].

3.4. Time-resolved Linear Dichroism Spectroscopy

In addition to the simultaneous detection of CD and CB in the ellipsometric measurement (null), there are also contributions from LD and linear birefringence (LB). These linear and circular effects measure the differences in the absorption coefficients and in the refractive indices for light linearly polarized in the direction parallel and perpendicular to the orientation axis and for LCP and RCP, respectively. However, unlike CD and CB, which are effects present in only chiral samples or under the influence of a magnetic field, LD and LB are more prevalent and have large effects in ordered samples [76]. Order in an initially isotropic sample can be introduced by the technique of photoselection [77] using linearly polarized lasers such as those used to initiate a biochemical or protein folding reaction. Molecules whose transition dipoles are aligned along the same polarization axis as that of the excitation laser become preferentially excited, forming an oriented subset. If there is a difference between the index of refraction or extinction coefficient of the photoproduct and the initial species, then the sample will be linearly birefringent or linearly dichroic, respectively. With time, the photoselected population undergoes reorientation facilitated by thermal rotational diffusion, by changes in the environment of the chromophore, or by relaxation of the photoproduct back to the initial state. Depending on the relative rates of thermal reorientation and photoproduct relaxation, TRLD measurements can provide information on the transition dipole orientations of the transient species or on the rotational diffusion parameters of biomolecules [16,78]. Thus, although LD is considered an artifact in the context of the experimental techniques discussed above, it is also a powerful tool for the study of the nature of molecular structure, conformations, and orientations.

Linear dichroism is observed in molecules that absorb two orthogonal linearly polarized light beams to different extents. A simple way to obtain TRLD spectra is the direct measurement of absorption signals oriented parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the polarization axis of the pump laser. This method is ideal for large TRLD signals, but in cases where LD is small a more sensitive measurement is preferred. One method that improves the signal-to-noise ratio uses a PEM to modulate the polarization state of light between two crossed linear polarizers [79]. However, like the conventional CD approach, the PEM modulation frequency limits the time resolution of the LD measurement. Improved time resolution was made possible when the PEM was coupled to a Q-switched Nd:YAG laser with a high repetition rate [43] and higher

sensitivity was achieved by monitoring the photoinduced transmission between crossed polarizers [80]. Although the latter approach is the most sensitive for TRLD because it uses the null measurement, it is confounded by the presence of LB.

In 1994, this laboratory introduced a technique that provides ultrasensitive measurements of absolute linear dichroism with minimal interference from artifacts and with nanosecond time resolution [36]. This quasi-null TRLD measurement is essentially a modification of the TRORD apparatus and it is based on the fact that a linear dichroic sample rotates the polarization plane of linearly polarized light. Because this is also true for CB, the TRORD apparatus actually measures a linear combination of CB and LD. Although potentially problematic for ORD measurements of photoinitiated reaction at times shorter than the chromophore diffusional reorientation time, this can be strategically addressed by tuning the polarization of the probe beam. That is, one effect may be selectively enhanced over the other by varying the angle between the pump and probe polarization vectors. When the probe beam polarizers, P1 and P2, are oriented parallel and perpendicular to the polarization axis of the pump beam pure CB can be measured. However, to obtain pure LD the probe polarizers are oriented $\pm 45^\circ$ to the laser polarization.

TRLD experiments in this laboratory initially used the direct absorption method to probe the transition dipole orientations of the early rhodopsin photolysis intermediates [81]. However, later studies used the ultrasensitive TRLD technique to study the photocycle of bacteriorhodopsin in purple membrane patches, the results demonstrating that during transition from the K to L intermediate (50 ns to 50 μ s) the retinal chromophore rotates less than 8° relative to the unphotolyzed chromophore [36,82], and the rebinding kinetics of sickle hemoglobin polymers, where the rate of photodissociated CO ligand recombination to the polymers was found to be ~ 1000 times slower than the rate for CO rebinding to T-state hemoglobin monomers [83].

4. Temperature-Jump Time-resolved Optical Rotatory Dispersion Spectroscopy

In studies of protein folding, the availability of fast detection methods would have minimal impact if it were not also possible to initiate the reaction of interest on an equally fast or faster time scale. Although there are well-established fast biochemical reaction initiation methods, such as ligand photolysis, photoreduction, and chromophore photoisomerization, their use in studies of folding is limited to only the proteins that have either an exogenous or endogenous photoactive group [84]. One of the advantages of the T-jump trigger is that it is a more general way to initiate folding in proteins/peptides. Another advantage of the T-jump trigger is that for many proteins it is possible to study folding kinetics without the use of denaturant to rapidly perturb the equilibrium between folded and unfolded states. The T-jump trigger has been coupled with fast detection methods, such as TROD, time-resolved fluorescence, TR^3 , and TRIR, to probe the dynamics of protein folding/unfolding during the submillisecond (burst phase) [1,3]. The remainder of this article will focus on the T-jump TRCD/TRORD apparatus that was recently developed in this laboratory to provide information that complements the current understanding of protein folding from the perspective of global secondary structure changes.

The T-jump TRCD/TRORD configuration discussed here is not the first attempt at such an instrument. The earliest T-jump CD (TJCD) system was developed in the 1970s, with single wavelength detection and $\sim 300 \mu$ s time resolution [31]. A capacitive electrical discharge was used in those studies to achieve a T-jump within $\sim 5 \mu$ s. However, some protein/peptide structural changes are expected to occur faster than the time resolution of that T-jump. Other ways by which a rapid change in temperature can be achieved are the absorption of light by

either a dye [85] or water [86–88]. Although the time resolution of the T-jump generated by dye absorption is on the picosecond time scale, the dye can potentially interfere with protein folding/unfolding processes. This possibility for interference is also true for the high salt concentrations required by the method of capacitive electric discharge [89]. Thus, the most common way to obtain a rapid T-jump is by direct absorption of IR light by water in aqueous samples. Using a high-powered laser at a wavelength of 1.4 to 2 μm , it is possible to induce a 10 K or greater increase in temperature on a time scale within the laser pulse width.

Subsequent to the TJCD apparatus discussed above, this laboratory developed a T-jump TRCD/TRORD system that was initially coupled to single wavelength OR/CD measurements [41]. Several significant modifications ensued to improve the system design. First, the implementation of multi-wavelength detection was important because it allowed clearer distinction between optical artifacts and phenomena of interest. Second, ORD became the predominant probe. In general TRCD spectroscopy is very sensitive to birefringence artifacts (e.g., cell window strain and photoselection) because these effects couple with CD measurements to first order. For this reason, in T-jump TRCD experiments, the birefringence artifact of most concern is that which arises from the rapid temperature change. This artifact is mitigated in ORD spectroscopy because birefringence only couples to this measurement in second order. Finally, the probe beam in the TRORD apparatus was modified for better pump-probe overlap (see section 4.2).

4.1. T-jump Trigger

4.1.1. Raman generation of the T-jump pulse—The T-jump trigger has been used successfully with several time-resolved detection methods to initiate protein folding reactions [3]. A 10 K or greater change in temperature is achieved with a 1560 nm heating pulse that is generated by the use of optical parametric oscillator crystals [90] or stimulated Raman scattering (SRS) [86,91] of D_2 gas. The SRS approach, which is used here, is very similar to that described previously [92]. In the T-jump TRORD system, design of the rapid heater, as shown in Figure 3, is based on passing the output of a Nd:YAG laser (7-ns pulse width) through two 2 m Raman shifters, each filled with about 400 PSI D_2 gas. The Nd:YAG is protected from potentially damaging laser back-reflections by the first optical component, an optical isolator, which comprises a 1064 nm thin film polarizer and a Faraday rotator. A 50/50 beam splitter generates two lines of 1064 nm light and sends each one to a telescope that compresses the beam diameters by a factor of about 2. Using 1064 nm high efficiency reflectors, the beams are directed through the shifters and the 1560 nm beams are selected with short wave pass dichroic beam splitters that transmit the 1064 nm beams to beam dumps and reflects the 1560 nm beams to another set of telescopes. The two 1560 nm beams arrive at the sample via 1560 nm high efficiency reflectors along counterpropagating paths. At the sample, the diameters of the 1560 nm beams are controlled by the telescopes.

4.1.2. Temperature jump – size, gradients, and artifacts—In a 300 μm path length cell, the use of a single beam to induce a change in temperature would generate an approximately 45% temperature gradient through the cell. The use of two counterpropagating heating beams of similar intensities reduces the temperature gradient to less than 5% [42,92]. Typically, the two 1064 nm beams pump the Raman shifters with ~ 230 mJ pulses of 1064 nm light, which is sufficient to generate ~ 55 mJ of 1560 nm light. This light, the first Stokes line, is absorbed by the overtone of the OH-stretch of water with an absorption coefficient of about 9.6 cm^{-1} [93]. By using the absorption of phenol red dye at different temperatures, a calibration curve is calculated and used to determine the size of the T-jump. The change in the phenol red absorption induced by each counterpropagating beam is of the same magnitude within 10%.

The dimensions of each 1560 nm beam, as well as the probe beam, were approximated by using vertical and horizontal knife-edge measurements [94]. The current configuration is such that there is a ~4:1 heating (ca. 2 mm) to probe beam (0.5 mm) diameter ratio. This ratio addresses the issue of a possible temperature gradient across the probe beam. That is, if the probe and heating beams are similar in dimension then there will be a significant temperature gradient from the center of the probe beam. Given such a gradient, the probe beam may either sample a heterogeneous solution of unfolded protein ensembles with different kinetics or it may sample different percentages of unfolded ensembles with the same kinetics. With the heating:probe beam ratio of 4:1, the probe beam will be able to see the region closer to the peak of the Gaussian-shaped heating beam for a homogeneous, thermally distributed profile and for measurements of more accurate kinetic data.

A significant advantage of using a probe source with multi-channel detection is that artifacts can more readily be distinguished from the spectrum of interest. One of the main artifacts that can accompany the rapid change in temperature is nucleation and growth of bubbles, or photoacoustic cavitation. Because the dynamics of cavitation have been characterized by time-resolved absorption methods - initiation within 250 ns, maximum growth around 2.5 μ s, and decay by 10 μ s after the initial laser pulse [95] – it is easy to identify and therefore, to minimize the artifact. For far-UV TRORD measurements, the response of buffer solutions to the T-jump showed baseline offsets that did not correspond in time with those reported for bubble formation and decay [42]. However, the TRORD studies (on different buffers) demonstrated that these offsets are sufficiently reproducible that they can be subtracted from the protein data. Figure 2e shows unfolding of RNase A after a ~10 K change (313 to 323 K) in temperature. The far-UV TRORD data indicates that unfolding occurs within the earliest measured time point, 35 ns, of the experiment. Subsequently, the TRORD signal does not change until after about 1 ms, when the signal decreases concomitantly with the dissipation of the heat introduced by the T-jump. The about 12% change in ORD signal is smaller than expected from equilibrium ORD measurements (17% decrease in signal from 313 to 323 K) and is attributed to the similar sizes of the heating and probe beams. With improvements to the T-jump TRORD apparatus preliminary TRORD measurements show an approximate 17% decrease in signal at 100 μ s after a 10 K T-jump, as shown in Figure 2f. Those preliminary data are noisy because the signals represent 80 averages in comparison to the 700 averages collected for the signals shown in Figure 2e. The modifications recently made to the T-jump TRORD system will improve the signal to noise of the experiment.

4.2. T-jump TRORD apparatus

The TRORD configuration that is coupled to the T-jump trigger has been modified from the apparatus introduced in 1995. Reduction of the probe beam diameter allows measurements of small sample volume and is also critical to optimizing the overlap with the heating beam and minimizing the temperature gradient across the probe beam. The current far-UV TRORD configuration is shown in Figure 4, with the main modification being the use of two UV-enhanced aluminum spherical mirrors (UV-SM). As a result, the initially unpolarized light travels from the flash lamp to the first polarizer and then to the first UV-SM, where the ~6-mm diameter probe beam is focused down by a factor of about 20. The second, collection UV-SM is positioned off-axis to direct the probe beam to the second polarizer. With a turning mirror, the probe beam then is focused onto the slit of a spectrograph that is equipped with a 600-groove/mm (200 nm blaze) grating for a ~100 nm spectral range and detected with a CCD detector.

5. Conclusion

Nanosecond time-resolved polarization spectroscopy has been applied to the study of several biochemical processes. More recently, nanosecond TRORD, TRCD, and TRMCD have contributed significantly to our understanding of protein folding in cytochrome *c*, RNase A, and azobenzene cross-linked peptides. For example, far-UV TRCD (Figure 2b) and visible TRMCD (Figure 2e) studies have presented the groundwork for conformational heterogeneity during the earliest stages of folding in reduced cytochrome *c*. The far-UV TRCD/TRORD studies (Figures 2b and 2c) identified unusual kinetic behavior as a function of denaturant for the ultrafast secondary structure formation phase [59, 70, 71], pointing to a molten-globule-like submillisecond folding intermediate in this protein [96]. In parallel, the structure sensitivity of TRMCD spectroscopy allowed detection of conformational ensembles with different tertiary constructs and kinetically decoupled folding pathways in cytochrome *c* [15]. This result provided the first direct experimental support for the multiple kinetic pathway prediction of the funnel landscape model of protein folding. Another significant contribution from the TRMCD methods was the measurement of the conformational diffusion time [21] that defines the time scales for the classical and energy landscape models of folding [97].

To provide information that complements the current understanding of protein folding from the perspective of global secondary structure changes and to study a larger number of proteins, a T-jump TRCD/TRORD apparatus was developed in this laboratory. The application of the T-jump TRORD apparatus to RNase A (Figure 2d) and cytochrome *c* has demonstrated that nanosecond secondary structure changes induced by a rapid perturbation of temperature can be detected by this method [42]. Current efforts have been focused on improving the signal-to-noise of the apparatus and on understanding the α -helix to β -sheet transition in poly-L-lysine.

Acknowledgments

This research was supported by Grant EY00983 and EB02056 from the the National Institutes of Health.

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Figure 1.

Time-resolved Circular Dichroism Apparatus. The TRCD apparatus is based on a quasi-null approach that allows measurement of the sign and magnitude of CD spectra. A strain plate to introduce a slight phase retardance that converts linearly polarized light to highly eccentric LEP and REP. Because elliptically polarized light comprises left and right circularly polarized light of different magnitudes, a circularly dichroic sample will either add to or subtract to the ellipticity of the LEP and REP.

Figure 2.

Results from selected time-resolved polarization studies. a) TRCD studies of protein function. Upon photoinitiation of the phytochrome A switch-on (Pfr, white circles) to switch-off (Pf) reaction, the N-terminal α -helical unfolding process was monitored with CD in the far-UV region and assigned to a 310 μ s time constant. The spectrum measured 5 ms after photoinitiation can be overlaid with the spectra of Pr obtained by both CW and laser illumination. b) Far-UV TRCD studies of protein folding. Photoreduction-triggered folding of reduced cytochrome *c* in 3.3 M GuHCl shows formation of the native protein CD spectrum (grey-filled circles) by 500 ms from the initially unfolded oxidized state (white circles). The spectra shown were measured at 3 and 7 μ s, 1, 10, 50, 100, 320, and 500 ms. Analysis of the data indicated that the

reduced protein folds in two major phases, with time constants of about 5 μ s and 100 ms. The 210 nm band is not observed here because of significant light absorption by GuHCl in that region. c) Soret TRMCD studies of protein folding. The data describes the dynamics of heme ligation events during CO-photodissociation-initiated reduced cytochrome *c* folding in 4.6 M GuHCl. TRMCD spectra shown here were calculated from TRMORD signals measured from 330 ns to 25 ms, with 4 times / decade. Global kinetic analysis of the TRMCD data reports spectral changes with time constants of 3, 5, 300, and 700 μ s. d) Far-UV TRORD studies of protein folding. Within the first measurement at 320 ns, the TRORD signal increases by about 20% and does not show further changes until after about 1 ms. The data shown here were obtained at 320 and 500 ns, 1.5 and 500 μ s, 100, 500, and 800 ms after folding of reduced cytochrome *c* in 4 M GuHCl was initiated by rapid electron transfer. e) T-jump TRORD studies of protein unfolding. Unfolding of RNase A (pH 3) is very fast, with the 12% decrease in TRORD signal at 228 nm observed at 35 ns maintained at 250 ns, 5 μ s and 1 ms. The change in ORD signal upon a 10 K temperature change from 313 to 323 K is smaller than expected (17%) from equilibrium thermal denaturation studies. This discrepancy is attributed to the similar sizes of the heating and probe beams. f) Protein studies with an improved T-jump TRORD system. Recent modifications to the T-jump TRORD system has allowed more flexibility in the sizing of the heating pump beam. The preliminary data shown here are considerably noisier because the signals reflect 80 averages in contrast to the previously accumulated 700 averages in (e). However, the expected 17% decrease in TRORD signal is clear in the signal measured at 100 μ s. In a, b, d, e, and f, all initial and final states are represented by white-filled or grey-filled circles, respectively. For all, the arrows indicate changes with time.

Figure 3.

Raman generation of the temperature-jump pulse. In the T-jump TRORD system, the design of the rapid heater splits the fundamental output of a Nd:YAG with a 50:50 beam splitter and passes each approximately 230 mJ beam through separate Raman shifters filled with 400 PSI D₂ gas. The generated 1560 nm light from each Raman shifter is selected with short wave pass dichroic beam splitters and the two heating beams arrive at the sample along counterpropagating paths (see Figure 3).

Figure 4.

Time-resolved Optical Rotatory Dispersion Apparatus. The TRCD (Figure 1) and TRORD systems are fundamentally different by one element, the strain plate. A reference rotation, β , is introduced into the probe beam polarization axis by rotating the first polarizer $\pm\beta$ relative to the cross position of the two polarizers. The CB from a chiral sample will add to or subtract from β . The TRORD configuration that is coupled with the T-jump trigger uses UV-enhanced aluminum spherical mirrors to focus the probe beam down by factor of about 20. This geometry allows measurement of small sample volumes and is critical to optimizing the overlap of the probe and heating beams. The latter minimizes the temperature gradient across the probe beam.