Structural Studies on Some Dityrosine-Cross-Linked Globular Proteins: Stability Is Weakened, but Activity Is Not Abolished†

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ABSTRACT: We have carried out conformational and stability studies on three proteins that have previously been shown to undergo dityrosine (DT) cross-linking. They include the monomers and dimers of DT-cross-linked calmodulin and the dimers of bovine pancreatic ribonuclease A and bovine eye lens γB-Crystallin. In each of these cases, we find the secondary and tertiary structure of the parent protein to be largely maintained. The DT dimer is, however, weaker than the parent. In this sense, the properties of these DT dimers are somewhat similar to those of glutaraldehyde-cross-linked protein crystals. In contrast, the intramolecularly DT-linked monomeric protein that we studied (DT monomer of calmodulin) is seen to have suffered greater changes in its conformation and stability. These results gain significance in light of the growing identification of DT formation as a marker of oxidative stress, aging, and disease.

Oxidative modifications in proteins are increasingly used as markers of aging, stress and pathogenesis, and environmental and behavioral assault such as radiation- or smoke-induced cell damage (*1*−*5*). Such modifications to a protein occur most readily to the side chains of amino acid residues Cys, Met, Trp, Tyr, His, and Phe. The non-tryptophan fluorescence (emission around 400−440 nm, upon excitation in the 310−360 nm region) seen in proteins is attributed to the oxidation products of Trp (e.g., kynurenines, hydroxy quinolines, and anthranilates), of Tyr (dityrosine), and of the Maillard reaction products (between reducing sugars and free amino groups of proteins, e.g., β-carbolines and pyrrolines). Covalent cross-linking of proteins is known to be mediated by disulfide bridges, His−Lys linkages, and DT[1] (Figure 1), which can be formed either photochemically (*6*) or in the dark through enzymatic and reactive oxygen species-mediated coupling (*7*).

With the growing identification of protein oxidation as a pathological manifestation, interest in DT cross-linking has also grown in recent years. DT is now known to occur in amyloid fibril formation (*8*), Parkinson's disease (*9*), atherogenesis caused by hemodialysis (*10*), atherosclerotic plaque formation (*11*), cerebrospinal fluid damage (*12*), lipofuscin granules in the brain (*13*), epidermal growth factor receptor dimerization in epidermoid carcinoma (*14*), cataract of the eye lens (*15*, *16*), aging (*17*), and oxidative stress such as during exercise (*18*). Dityrosine levels in body fluids (*19*), as well as immunohistochemical and ELISAs for dityrosine (*17*), have been suggested as in vivo biomarkers of oxidative stress in aging and in disease.

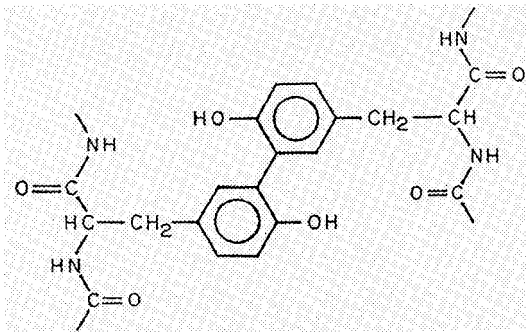

FIGURE 1: *o*,*o*′-Dityrosine (DT) cross-link.

While the formation of DT linkages in proteins has been documented, its consequences on the structure and biological function of the modified proteins are yet to be fully understood. In the cuticlin protein of *Caenorhabditis elegans* (*20*), cell wall proteins of yeast (*21, 22*) and bamboo shoots (*23*), and parchment collagen (*24*), DT cross-linking is believed to be beneficial in terms of structural rigidity and strength; with other proteins, particularly globular ones, cross-linking could lead to alterations in conformation, ligand binding, and biological activity.

In terms of this relationship, pioneering and instructive studies have been conducted by Malencik and Anderson (*6*, *25*−*28*) on the intramolecularly DT-linked monomeric form of calmodulin as well as its DT dimers and higher oligomers. They find the intermolecular DT dimer behaves like the parent monomer in its ability to bind smooth muscle light chain kinase, and also like calcium, though the binding here is weaker and occurs over a range of concentrations (*26*). In

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[1] Abbreviations: DT, dityrosine; GdmCl, guanidinium chloride; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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contrast, the intramolecularly DT-linked calmodulin is unable to stimulate the *p*-nitrophenylphosphatase activity of calcineurin and displays greatly reduced affinity for smooth muscle myosin light chain kinase and calcium. Aeschbach et al. (*29*) have previously studied the residual enzymatic activity of ribonuclease A, trypsin, and chymotrypsin subjected to enzymic oxidation. They find that while DT-containing trypsin and chymotrypsin lose complete enzymatic activity, the activity of DT-containing RNase A is unaffected. A recent report by Lardinois et al. (*30*) has shown that the DT dimer of lactoperoxidase retains the full catalytic activity of the parent monomer. Dimerization does not affect the binding and catalytic regions of the molecule; however, oligomerization of the enzyme alters its physical properties and perhaps its ability to interact with macromolecular substrates. Bodaness et al. (*31*) had earlier shown that γB-Crystallin undergoes DT-cross-linking upon exposure to H_2O_2, mediated by the heme undecapeptide from cytochrome *c*. Subsequently, we have shown (*32*) that DT dimerization of the eye lens protein γB-Crystallin does not alter its conformation in a major manner, but reduces its solubility and native structural stability.

In light of the increasing number of reports on DT cross-linking and its association with pathology, we have undertaken a study of the structural and stability features of several purified and well-characterized DT-linked proteins, namely, (a) the enzyme bovine pancreatic ribonuclease A (RNase A), (b) the calcium-binding protein bovine calmodulin involved in signal transduction pathways, and (c) γB-Crystallin, a structural component of the mammalian eye lens.

MATERIALS AND METHODS

Proteins. γB-Crystallin was isolated from fresh calf lenses and purified using the procedures described previously (*32*). Calmodulin was isolated from bovine brain and purified by using the method of Gopalakrishna and Anderson (*33*). The calcium-free protein was obtained by 10% trichloroacetic acid precipitation with solutions containing 10 mg/mL protein in the cold, followed by centrifugation. The resulting pellet was dissolved in 1 M tris(hydroxymethyl)aminomethane (pH 8.0). After this procedure was repeated one or two times, the sample was dialyzed against Chelex-100-treated buffer [20 mM HEPES (pH 7.0)] and finally against Milli-Q water. RNase A was obtained from Sigma Chemical Co. as a lyophilized powder and was further purified by ion exchange chromatography on a Pharmacia Mono-S 10/16 column using acetate buffer. Tris and Mops buffers were prepared with distilled water that had been purified with a Milli-Q reagent water system and treated with Chelex-100. Reagent grade or the best available grade chemicals were used throughout.

Preparation and Purification of the DT-Linked Proteins. The DT-linked dimer and the DT-linked monomer of calmodulin were prepared by direct UV irradiation as described in detail by Malencik and Anderson (*6*, *26*) and purified using their protocol. The DT-linked dimers of γB-Crystallin and RNase A were generated by riboflavin-mediated sensitization. A solution of γB-Crystallin (1 mg/mL) containing 30 μM riboflavin as the sensitizer was placed in the sample cell of a Hitachi F-4000 spectrofluorimeter and irradiated at 445 nm for 5 min, with an excitation bandpass of 20 nm. Previous measurements from our laboratory have estimated the radiation flux under these experimental conditions to be 10^{14} photons/s, or 0.2 mW/cm^2 (*34*). The reaction was stopped by the addition of dithiothreitol (DTT) to a final concentration of 10 mM. RNase A was irradiated similarly, and the reaction was stopped by rapid desalting on a PD-10 column (Pharmacia). Sodium phosphate buffer (pH 7.4) was added to both the proteins to a final concentrations of 10 mM. After concentration in an Amicon ultrafiltration unit at 4 °C, the DT dimer and "irradiated monomer" were resolved by gel filtration on a HR 10/60 Superdex-75 column [which was equilibrated in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl and 1 mM DTT for γB-Crystallin separation].

The separated proteins were concentrated and dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl for all studies unless specified. Protein concentrations determined by the Bradford method (Bio-Rad kit) using native calmodulin, RNase A, and γB-Crystallin as standards for the respective proteins were corroborated by amino acid analysis.

Preparation of the Dityrosine Standard. Dityrosine was prepared by the method of Malencik et al. (*35*). The purity of the compound was checked by reverse phase HPLC and mass spectrometry.

Amino Acid Analysis. The amino acid compositions of control and irradiated proteins were determined with an LKB (model 4151 alpha plus) amino acid analyzer connected to an LKB 2221 integrator unit. The samples were hydrolyzed in evacuated sealed tubes under acidic conditions (6 N HCl) at 105 °C for 24 h. The amino acids in the hydrolysate, after separation on a cation exchange column, were detected at 570 and 440 nm after postcolumn derivitization with ninhydrin. Norleucine (10 nmol) was added to each sample prior to hydrolysis for the estimation of recovery after hydrolysis. The number of DT cross-links formed were calculated, based on the values determined by amino acid analysis using the dityrosine standard, the number of Tyr residues found in the control, and the number present per protein molecule after oxidation (*36*, *37*). The analysis of native, DT dimer, and intramolecular DT monomer of calmodulin yielded values similar to those obtained by Malencik and Anderson (*6*, *26*). The amounts of DT in the dimer and monomer of calmodulin were estimated from the area under the peak corresponding to the dityrosine standard and were found to be approximately 0.9 and 0.75 mol/mol of the DT dimer and DT monomer, respectively. There was no significant decrease in the methionine and histidine contents of the DT dimer and DT monomer, respectively.

SDS−PAGE and Densitometric Scanning. Reducing polyacrylamide gels (10%) were run in the presence of SDS and β-mercaptoethanol, to determine the degree of non-disulfide cross-linking in the irradiated protein. The gels were stained with Coomassie Blue and scanned on a Molecular Dynamics computing densitometer (version 3.22).

Measurement of the Enzymatic Activity of RNase A. The activity of the control and modified proteins was measured by the method of Crook et al. (*38*). The increase in optical density at 284 nm that occurs upon addition of 10 μg of either native RNase A, the "irradiated monomer", or the DT-linked dimer of RNase A to a solution of 0.1 mg/mL cyclic CMP in 100 mM Tris-HCl buffer (pH 7.13) (with the ionic strength adjusted to 0.2 M with NaCl) was monitored as a

function of time. The rate of increase in the optical density is the measure of enzyme activity. The percentage change in the activity of the DT dimer was calculated with respect to that of control RNase A.

Spectral Measurements. Fluorescence spectra were recorded with a Hitachi F-4010 spectrofluorimeter. Borate quenching of DT fluorescence (*25*), was studied by the addition of 200 μL of a 1 mg/mL protein solution to 1 mL of 0.5 M borate/boric acid (pH 8.7), and the excitation maximum was 320 nm. The control spectrum was acquired with protein in 1.5 M Tris-HCl buffer (pH 8.8). Quenching of the fluorescence of the crystallins was carried out using KI and acrylamide as quenchers, and the Stern−Volmer equation was used to the estimate fractional accessibilities (f_a values) of the fluorophores (*32*, *39*). Circular dichroism (CD) spectra were recorded using a Jasco J-715 spectropolarimeter. Far- and near-UV CD spectra were measured using 1 mg/mL protein solutions in the appropriate buffers, in 0.01 and 1.0 cm path length cells, respectively. Ellipticities were expressed as $[\theta]_{MRW}$ where MRW is the the mean residue molecular mass, taken to be 115 Da. Mass spectra were recorded at the ESI-MS facility of the Indian Institute of Science (Bangalore, India) and the Indian Institute of Chemical Technology (Hyderabad, India). The proteins were desalted for the analysis by reverse phase HPLC on a Bio-Rad 250 mm × 4.6 mm C4 column using a linear gradient of water and acetonitrile containing 0.1% trifluoroacetic acid or by passing them through a PD10 Pharmacia desalting column equilibrated in MQ water.

Denaturation Studies. (1) RNase A. In the study of the thermal denaturation of RNase A, the change in protein absorbance with changing temperature was monitored at 287 nm on a Shimadzu UV−visible spectrophotometer equipped with a thermostatically controlled cell holder. The internal cuvette temperature was monitored using a microthermocouple thermometer (Physitemp). Typically, the various proteins (0.3 mg/mL) in 50 mM sodium cacodylate buffer (pH 6.5) were used. The rate of heating was set at 1 °C/min, and the reversibility was measured by monitoring the cooling curves, which could be superimposed on the heating curves. For the chemical denaturation studies of RNase A, the various proteins were incubated overnight at room temperature in varying concentrations of GdmCl in 50 mM sodium cacodylate buffer (pH 6.5). The change in tyrosine fluorescence was monitored at 305 nm upon excitation at 268 nm. The concentration of the stock solution of GdmCl was calculated from refractive index measurements.

(2) γB-Crystallin. For the thermal denaturation studies of γB-Crystallin, the concentration of the protein solution was 0.1 mg/mL in 20 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl. The shift in the tryptophan fluorescence band maximum of the proteins was measured upon excitation at 295 nm. The excitation and emission monochromators were set at 5 and 1.5 nm, respectively. The temperature was maintained using a Julabo thermostated water bath and the sample temperature measured using a Physitemp microthermocouple thermometer. The temperature-dependent change in the fluorescence emission maximum was used as the parameter to estimate the extent of denaturation.

(3) Calmodulin. Thermal and chemical denaturation studies with calmodulin were carried out using circular dichroism. The CD spectra of calmodulin, its DT monomer, and the DT dimer were recorded in 20 mM Hepes buffer (pH 7.0). For the calcium titration studies, the changes in the secondary and tertiary structures of calmodulin and its DT dimer and DT monomer were monitored by following the circular dichroism signals at 222 and 262 nm, respectively, as a function of total calcium concentration. Calcium was added in aliquots, using a Hamilton syringe, from a 0.5 M $CaCl_2$ stock solution after diluting it 1:100 or 1:10 for studying the secondary structural changes and 1:5, or as such, for studying the tertiary structural changes. Thermal and urea denaturation studies were carried out by monitoring ellipticity changes at 222 nm as a function of temperature and urea concentration, respectively, in both the absence of calcium (with 2 mM EDTA), as well as the presence 0.5 mM calcium. All ellipticity values are expressed as mean residue weight $[\theta]_{MRW}$ (degrees per square centimeter per decimole).

RESULTS AND DISCUSSION

We have studied the conformational and structural properties of DT dimers of γB-Crystallin, RNase A and calmodulin, and the DT monomer of calmodulin. There are several ways in which the dityrosine moiety can be produced, e.g., through radiolysis (*40*), direct UV irradiation at 280 nm (*6*, *34*), Fenton reaction (*1*, *40*), Fe(IV)−porphyrin radicals (*30*, *37*), superoxide radicals (*7*), and photodynamic methods (*32*, *41*). Our attempts of generating DT using methylene blue yielded negative results; previous studies (*41*, *42*) using Rose Bengal also did not result in any DT formation. This suggests that sensitizers which operate predominantly through the type II pathway (largely yielding singlet oxygen as the oxidant) are not very efficient in catalyzing DT production. Hence, we used riboflavin (which operates by the type I pathway as well, yielding carbon and oxy radicals) to photodynamically generate DT in proteins. The results of our investigation on the purified DT-linked proteins are discussed below.

DT Dimer of RNase A. Native RNase A shows its tyrosine fluorescense emission maximum at 305 nm, upon excitation at 280 nm. Upon riboflavin-mediated photosensitization of RNase A, a decrease in the intensity of the Tyr fluorescence at 305 nm, with a corresponding increase in the intensity of DT fluorescence at 410 nm, is observed (Figure 2a). Reducing SDS−PAGE (Figure 2b) of RNase A irradiated for various periods of time shows the time-dependent formation of dimer(s) and higher oligomers. Dimers are seen to predominate after irradiation for 5 min. These dimers (peak I) were resolved from the irradiated monomeric fraction (peak II) of RNase A by gel filtration chromatography on a Superdex-75 column (Figure 2c). Most of the DT fluorescence was recovered in the dimeric fraction (peak I) with a negligible amount in the irradiated monomer (peak II). This observation is similar to what has been previously reported in the literature for the enzymatic oxidation of RNase A (*29*). The DT fluorescence of both the dimer and the monomer was quenched by borate (Figure 2d). Table 1 shows the amino acid analysis of the purified dimer, irradiated monomer, and control RNase A. DT was present at a level of 0.75−0.8 mol/mol of the dimer and 0.05−0.1 mol/mol of the irradiated monomer. Apart from the tyrosine content, which decreased by 20% in the irradiated monomer and 40% in the dimer, and the methionine content which decreased by 10% in both, the compositions of the rest of the amino

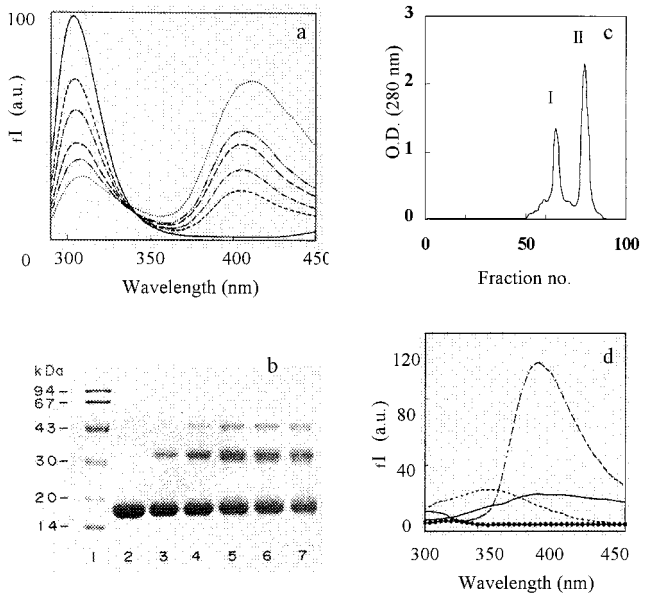

FIGURE 2: (a) Generation of DT fluorescence in RNase A. The tyrosine fluorescence decreases with a concomitant increase in the DT fluorescence with time: irradiation for 0 (—), 5 (- - -), 10 (-·-), 15 (— — —), 30 (—···—), and 60 min (·····), respectively. (b) DT dimers of RNase A form photodynamically: lane 1, molecular mass markers; lane 2, unirradiated RNase A; lanes 3−7, RNase A oxidized using riboflavin as a sensitizer, with irradiation times of 5, 10, 15, 30, and 60 min, respectively, at 445 nm. (c) Superdex elution profile of irradiated RNase A. Peaks I and II correspond to the dimer and irradiated monomer, respectively. (d) DT fluorescence spectra of the dimer (—·—) and the irradiated monomer of RNase A (—) in 1.5 M Tris buffer (pH 8.8). The quenched spectra of the dimer (- — -) and the irradiated monomer (●) in 0.5 M borate/boric acid (pH 8.7). I_f is the fluorescence intensity, expressed in arbitrary units (a.u.).

Table 1: Amino Acid Analyses of RNase A and Its Irradiated Monomer and DT Dimer[a]

amino acid	native protein	irradiated monomer	DT dimer
Asp	4.9 (5)	4.94	5.2
Thr	10.3 (10)	10.11	9.97
Ser	14.5 (15)	14.8	15.2
Glu	5.2 (5)	5.0	5.2
Pro	3.10 (3)	3.19	3.06
Gly	3.03 (3)	3.2	2.85
Ala	12.4 (12)	12.3	11.4
Val	8 (9)	8.15	8.07
Met	3.11 (4)	2.85	2.76
Ile	2.95 (3)	2.87	2.85
Leu	1.92 (2)	1.86	1.89
Tyr	5.7 (6)	4.5	3.4
Phe	2.97 (3)	3.03	3.11
DT	—	0.05	0.38
His	4.1 (4)	3.86	3.75
Lys	10.6 (10)	11.1	11.3
Arg	3.90 (4)	3.82	3.86

[a] Nanomoles obtained per 123 nmol of total values. Ideal values are given in parentheses.

acid residues were very similar to those of native RNase A. Though the irradiated monomer does not contain a significant amount of DT, amino acid analysis reveals a 20% decrease in the tyrosine content. This suggests loss of tyrosine by alternate photochemical reactions apart from DT formation. We have therefore used the "irradiated monomer" as a postirradiation control to determine the effect of DT dimerization on the conformation and stability of RNase A, as it might contain modifications other than DT.

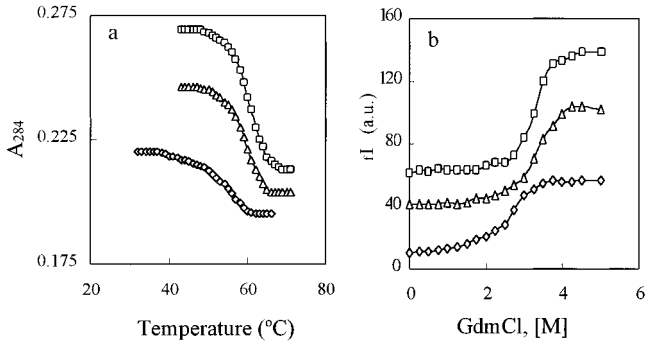

FIGURE 3: DT dimer of RNase A which is more susceptible to denaturation both thermally and isothermally. Panel a shows the thermal denaturation, expressed as absorbance at 284 nm, and panel b the GdmCl-induced denaturation, expressed as I_f, of native RNase A (□), its "irradiated monomer" (△), and DT dimer (◇).

The molecular mass of RNase A determined by electrospray analysis was 13 684 Da. No significant change in the molecular mass of the irradiated monomer upon irradiation (apart from adduct formation with sodium ions) was observed. The dimerized protein exhibited a molecular mass of 27 460 Da (the actual mass is 27 368 Da, the extra 92 Da being due to formation of adducts with four sodium ions).

We measured and analyzed the CD spectra of RNase A to gain insight into the effect of DT dimerization on its secondary and tertiary structure. The results showed that the CD spectral features are not altered significantly upon dimerization, indicating that there are no major changes in protein conformation upon DT cross-linking. We did not, however, detect any CD signals characteristic of the DT moiety (data not shown). We next studied the effect of DT dimerization on its conformational stability by monitoring the decrease in the Tyr absorbance at 284 nm as a function of temperature. Native RNase A denatures in a cooperative manner with a T_m of 60 °C (Figure 3a). Interestingly, the purified DT dimer of RNase A is seen to denature at the lower temperature of 54 °C, and in a less cooperative manner. We have also investigated the GdmCl-induced denaturation of RNase A and its DT dimer. The change in tyrosine fluorescence intensity at 305 nm was monitored as a parameter for estimating the extent of unfolding. Native RNase A and the irradiated monomer of RNase A denature almost identically at >3 M GdmCl. The DT dimer of RNase A denatures earlier at 2.6 M GdmCl (Figure 3b). It is worth noting that the irradiated monomer denatures essentially in the same manner as native RNase A. The conformational destabilization observed in the DT dimer can therefore be attributed to the dityrosine-mediated dimerization.

With regard to enzymatic activity, Aeschbach et al. (*29*) have previously studied the activity of DT-cross-linked RNase A (containing a mixture of component dimers and monomers) and found that it does not alter significantly upon DT formation. When we monitored the enzymatic activity of the purified DT dimer of RNase A, we found that it was lowered by 25% as compared to that of native and the irradiated monomer of RNase A.

DT-Linked Calmodulin. Bovine calmodulin has two Tyr residues in its sequence, namely, Tyr99 and Tyr138. Malencik and Anderson (*26*) have shown that intermolecular DT cross-linking of calmodulin to form dimers requires the absence of calcium, and of the three theoretically possible

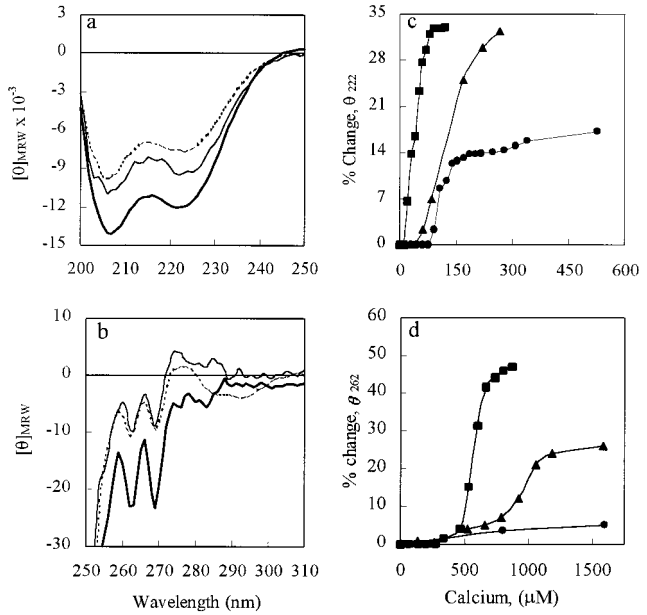

FIGURE 4: Near- and far-UV CD spectra of calmodulin which are altered upon DT cross-linking. Panel a shows the secondary structural region from 200 to 250 nm, and panel b shows the tertiary structural region from 250 to 310 nm of native calmodulin (−), its DT dimer (—), and its DT monomer (- − -). Panels c and d show the calcium-induced secondary and tertiary structural changes, respectively, plotted as a function of total calcium concentration in native calmodulin (■), its DT dimer (▲), and its DT monomer (●). $[\theta]_{MRW}$ is the mean residue molar ellipticity, expressed in units of degrees per square centimeter decimole. Protein concentrations for the secondary and tertiary structural changes are normalized to 17.5 and 200 μM (in terms of monomer units), respectively.

dimers, Tyr99−Tyr138 and Tyr99−Tyr99 dimers predominate. Intramolecular DT cross-linking, however, requires the presence of calcium (*6*), which brings the loop region of calcium binding site III (where Tyr99 is located) and IV (where Tyr138 is located) side by side in an antiparallel arrangement (*43*) to promote their cross-linking. The DT-linked dimer of calmodulin is shown to be more like native calmodulin in its ability to bind SMLK and calcium, though the binding here is weaker and occurs over a range of concentrations (*26*). In contrast, the intramolecular DT monomer of calmodulin is functionally more compromised; it displays a 280-fold-reduced level of binding with smooth muscle light chain kinase, and is unable to stimulate the *p*-nitrophenylphosphatase activity of calcineurin (*6*). We have carried out further physicochemical characterization of these DT-linked forms of calmodulin.

Panels a and b of Figure 4 show the far- and near-UV CD spectra of native calmodulin and its DT dimers and DT-monomer in the absence of calcium. The far-UV CD spectrum of calmodulin (Figure 4a) indicates that the level of predominantly α-helical secondary structure of calmodulin is reduced upon DT cross-linking, more so in the DT monomer. The near-UV CD spectrum of native calmodulin (Figure 4b) exhibits two prominent negative bands at 262 and 268 nm, attributable to the Phe residues (*44*). Both the DT monomer and DT dimer exhibit a marked reduction in the intensity of these two bands (Figure 4b). This reduction either could result from the increased conformational flexibility of some Phe residues as a result of conformational changes brought about by DT cross-linking or could be due to a loss of Phe residues as a result of oxidation. Amino acid analysis of the DT monomer and DT dimer revealed all seven Phe residues are in intact (data not shown). The decreased intensity of the Phe signatures in the DT monomer and DT dimer is thus likely to be due to the increased conformational flexibilty of the Phe residues.

The propensity of calmodulin to undergo large conformational changes upon calcium binding underlies its ability to mediate intracellular signal transduction. Calcium binding induces changes in both the secondary and tertiary structure of native calmodulin. It was of interest to investigate the effect of DT linkage on this ability of calcium to induce conformational changes in calmodulin. Panels c and d of Figure 4 show the ellipticity change at 222 nm (indicative of the secondary structure) and 262 nm (indicative of the tertiary strcuture), as a function of total calcium concentration. These structural changes (Figure 4c,d) in native calmodulin saturate at an ∼3-fold molar excess of calcium. The corresponding change in the dimer occurs at higher concentrations of calcium, probably reflecting calcium binding over an extended range of concentrations as reported previously (*26*). The DT monomer shows a smaller extent of secondary structural change and almost no tertiary structural change even in the presence of excess calcium.

Bovine calmodulin can be cleaved into two fragments by trypsin: the N-terminal half (residues 1−77) and the C-terminal half (residues 78−148). Martin and Bayley studied the calcium-induced structural changes in these two halves. They find that the C-terminal half contributes to nearly all the calcium-induced tertiary structural change in native calmodulin (*45*), while the N-terminal half makes a negligible contribution. Both Tyr99 and Tyr138, involved in the intramolecular DT linkage, reside in the C-terminal half of calmodulin. The intramolecular Tyr99−Tyr138 linkage probably renders the molecule insensitive to calcium-induced tertiary conformational changes. Could this be a reason for the drastically reduced ability of the DT monomer of calmodulin to bind smooth muscle light chain kinase in the presence of calcium?

Calmodulin, in the absence of calcium, is known to exhibit two premelting thermal transitions, in the 15−30 °C range and in the 40−60 °C range, before it denatures at >70 °C (*46*−*48*). In the presence of calcium, the main thermal transition is shifted to higher temperatures (*47*). Calcium stabilizes calmodulin against denaturation by temperature and chaotropic agents. Both DT dimers and DT monomers of calmodulin denature similarly in the presence of calcium, exhibiting a gradual change in ellipticity with increasing temperature (Figure 5a) or denaturant (Figure 5c). In the absence of the stabilizing influence of calcium, differences in their conformational stabilities become apparent. Figure 5d indicates the loss of about 30% ellipticity at low urea concentrations in native calmodulin, whereas the DT-linked proteins already have much lowered ellipticity values at 0 mM urea, as also observed in Figure 4a. The differences in the ellipticities of the DT dimer and DT monomer compared to that of native calmodulin reflect the structural differences between them. Only the first thermal transition observed at ∼25 °C (in native calmodulin in the absence of calcium) is lost in the DT dimer, while the second transition at ∼53 °C remains unaffected (Figure 5b). In the DT monomer, both these transitions are affected. The larger transition at 53 °C is lowered to 48 °C. The loss of the low-temperature

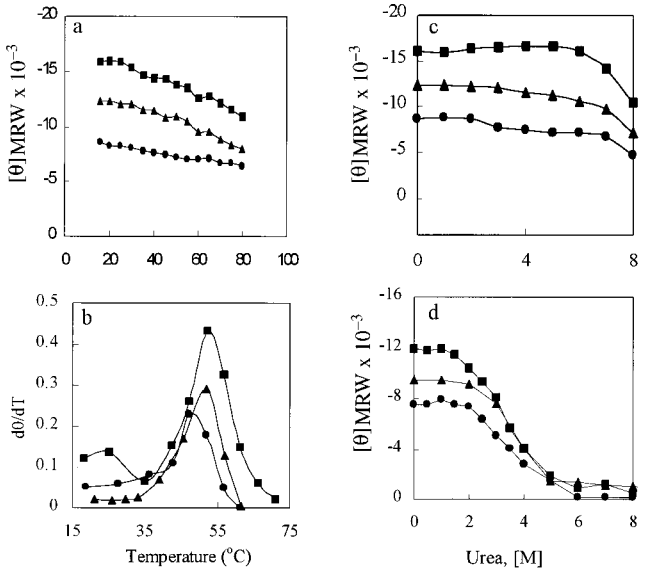

FIGURE 5: Ellipticity changes at 222 nm (a) in the presence of 0.5 mM calcium and (b) as the first derivative in the absence of calcium (with 2 mM EDTA) as a function of the temperature of native calmodulin (■), the DT dimer of calmodulin (▲), and the DT monomer of calmodulin (●). Urea-induced denaturation is plotted as ellipticity in the presence of calcium (c) and in the presence of EDTA (d) of native calmodulin (■), the DT dimer of calmodulin (▲), and the DT monomer of calmodulin (●). Ellipticities are expressed in the same units as in Figure 4.

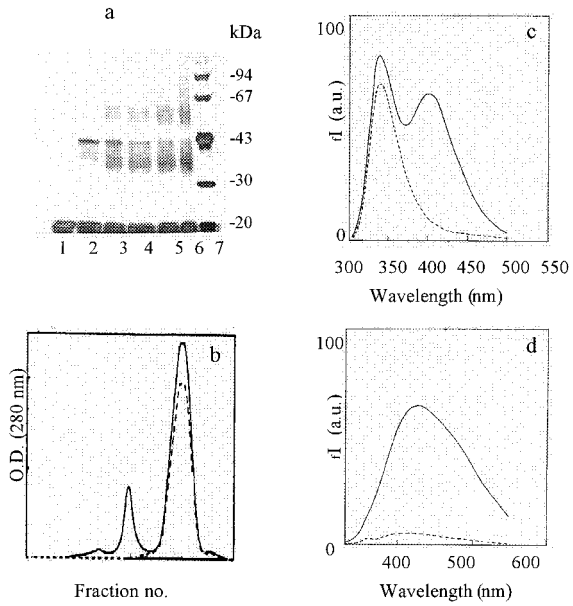

FIGURE 6: (a) Increase in reaction time leading to higher aggregates and to some degradation, too. Monitoring of the time dependence of the oxidation reaction of γB-Crystallin, with the assay and experimental conditions identical to those described above. The irradiation times are 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), and 30 (lane 6); control γB-Crystallin and the low-molecular mass markers are shown in lanes 1 and 7, respectively. (b) Superdex elution profiles of the control (- - -) and the irradiated (—) γB-Crystallin. Peaks I−III correspond to the trimer, dimer, and monomer, respectively. (c) DT fluorescence spectra of irradiated monomeric γB-Crystallin (- - -) and its DT dimer (—), seen in the 410 nm region (excitation at 280 nm). (d) Quenching of DT fluorescence by borate. The emission spectrum of the DT dimer of γB-Crystallin, obtained upon excitation at 320 nm (top curve), quenched and blue shifted in 0.5 M borate/boric acid (pH 8.7) (lower curve).

transitions in the DT monomer and DT dimer of calmodulin correlates with the structural differences observed at low urea concentrations.

In sum, we find that, by and large, DT linkage has the effect of weakening the structural order and stability of the calmodulin molecule, and the effect is more pronounced with the DT-linked monomer than with the DT dimer. These differences are especially evident in the calcium-free forms of the proteins.

DT-Dimerized γB-Crystallin. Having studied an enzyme and a protein important in calcium-mediated events, we then looked at the effect of DT dimerization on γB-Crystallin, a protein that plays a structural role in a tissue, namely, the mammalian eye lens. γB-Crystallin is the major constituent of the core of the lens, occurring there in concentrations as high as 500 mg/mL. Since its turnover rate is extremely slow, modifications that occur to the protein accumulate in time and contribute to the molecular pathology of the lens. We have previously studied the DT dimerization abilities of the various crystallins, with special reference to γB-Crystallin (*32*). The C-terminal Tyr175, which is well exposed to the solvent (*49*), might be involved in DT dimerization. Figure 6a shows the electrophoretic pattern of the sensitization reaction products of γB-Crystallin. It shows that 5 min of irradiation at 445 nm in the presence of 30 μM riboflavin produces significant amounts of the dimer, with traces of the trimer as well as some degradation products. We have separated the DT dimer of γB-Crystallin from the rest of the products by gel filtration chromatography on a Superdex-75 column (Figure 6b). Figure 6c compares the fluorescence emission spectra of the irradiated monomer and the DT dimer of γB-Crystallin. The DT dimer exhibits an additional emission maxima at 410 nm, which is quenched by borate (Figure 6d). Upon amino acid analysis, we found no significant changes in any other residues both in the dimer and in the irradiated monomer, except the oxidation of Met residues (ca. 30% decrease in the Met peak). Electrospray analysis of the native γB-Crystallin yielded a mass of 20 963 Da, in agreement with what is expected from the sequence; that of the irradiated monomer was 21 027 Da. The extra 64 Da appears to arise due to the oxidation of two of the seven Met residues in the molecule to the sulfone.

CD spectral measurements indicate that the secondary and tertiary structures of the DT dimer are quite similar to those of the parent native γB-Crystallin, as well as those of the irradiated monomer. However, an increased level of quenching of the Trp fluorescence by KI and acrylamide (*32*) in the DT dimer is an indication of subtle tertiary structural changes upon dimerization. The fractional accessibilities of the fluorescent residues (f_a values) of 0.84 and 0.90 seen with acrylamide and KI quenching of the DT fluorescence suggest that the DT linkage in the dimer is on the surface. This might be the reason the conformational features of γB-Crystallin are not altered substantially upon DT dimerization.

As with the DT dimers of RNase A and calmodulin, the DT dimers of γB-Crystallin also are structurally less stable than the monomer. The dimer denatures at 59 °C, as compared to native γB-Crystallin, which denatures at 66 °C. The dimer also precipitates out of solution at a lower temperature. Heating beyond 62 °C coagulates the DT dimer, while the parent γB-Crystallin does so only at >72 °C (*50*). This aggregation of the DT dimer of the Crystallin is of interest with respect to the issue of post-translational

modifications in the crystallins and their relation to lens transparency.

We have looked at the conformational and structural changes that occur to three proteins of diverse functions, upon DT dimerization. Though we have studied the properties of purified dimers, it should be noted that the dimers might be a mixture of DT-cross-linked isomers. Thus, the properties reported here could be a weighted average of the properties of the component isomers. We find no major conformational alterations upon DT dimerization, though the changes in calmodulin are more pronounced than in the other two proteins. As a general feature, however, DT dimerization tends to weaken the structural stability of these three proteins, a point of interest in the process of aging and in disease. The properties of these DT dimers are somewhat similar to those of cross-linked protein crystals, which retain substantial structural features and biological activity of the monomers (*51*−*53*). These properties are also reminiscent of those of the proteins whose surface Trp residues have been oxidized to produce *N*-formylkynurenine and kynurenine moieties (*54*); in these cases, too, the conformational features of the modified proteins are not substantially altered, though structural stability is weakened appreciably. The effects of DT dimerization on the function of enzymatic proteins are, however, varied. For example, DT-dimerized RNase A, lactoperoxidase (*29*), and calmodulin (*26*) retain much of their enzymatic activities. In contrast, DT-dimerized trypsin and chymotrypsin are enzymatically inactive (*29*). The effects of DT dimerization on the enzymatic and structural properties of proteins are therefore probably determined by the relative location of the tyrosines that are involved.

In comparison, the properties of the intramolecularly DT-linked monomer of calmodulin are more drastically altered. The C-terminal Tyr99−Tyr138 intramolecular linkage, which forms only in the presence of calcium, probably locks the C-terminal domain in a calcium-induced strained conformation.

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