Amino Acids (2003) 25: 233–247
DOI 10.1007/s00726-003-0014-z

Amino Acids
Printed in Austria

Dityrosine as a product of oxidative stress and fluorescent probe

Review Article

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Received December 20, 2002
Accepted May 8, 2003
Published online August 21, 2003;

Summary. Dityrosine can be a natural component of protein structure, a product of environmental stress, or a product of *in vitro* protein modification. It is both a cross-link and a fluorescent probe that reports structural and functional information on the cross-linked protein molecule. Diverse reactions produce tyrosyl radicals, which in turn may couple to yield dityrosine. Identification and quantitation of dityrosine in protein hydrolysates usually employs reversed phase high pressure liquid chromatography (RP-HPLC) or gas chromatography. RP-HPLC of protein hydrolysates that have been derivatized with dabsyl chloride gives a complete amino acid analysis that includes dityrosine and 3-nitrotyrosine. Calmodulin, which contains a single pair of tyrosyl residues, undergoes both photoactivated and enzyme-catalyzed dityrosine formation. Polarization measurements, employing the intrinsic fluorescence of dityrosine, and catalytic activity determinations show how different patterns of inter- and intramolecular cross-linking affect the interactions of calmodulin with Ca^{2+} and enzymes.

Keywords: Dityrosine – Tyrosyl radicals – Protein oxidation – Cross-linking – Calmodulin – 3-Nitrotyrosine

1. Introduction

The occurrence of dityrosine (Fig. 1) in proteins has a wide range of implications. The cross-linking of protein tyrosyl residues can be a normal physiological process, a pathological response to disease or environmental stress, and a useful tool for *in vitro* protein modification. Dityrosine is both cross-link and probe. The brilliant 400 nm fluorescence of dityrosine facilitates its detection and the performance of spectroscopic measurements to determine physical and functional properties of dityrosine-containing proteins. Our laboratory became interested in dityrosine when we encountered the ultraviolet irradiation-induced formation of dityrosine in the Ca^{2+} binding protein calmodulin (Malencik and Anderson, 1987). Dityrosine was discovered originally as a product of the horseradish peroxidase-catalyzed reaction between H_2O_2 and tyrosine (Gross and Sizer, 1959). Analysis of resilin, a structural protein that is found in insects and other arthropods, established dityrosine as a product of normal post-translational protein modifications (Andersen, 1963, 1964).

To date, there have been more than 245 published articles that deal with dityrosine. The five sections of this review deal with the diverse conditions associated with the occurrence of dityrosine in proteins, with analytical methods for the detection and quantitative determination of dityrosine, with the production and characterization of both intra- and intermolecularly cross-linked dityrosyl derivatives of calmodulin (Table 1), and with practical applications of the phenolic coupling of proteins. The usefulness of the intrinsic fluorescence of dityrosine for the physical and functional characterization of both the cross-links per se and the cross-linked proteins is demonstrated with our observations on calmodulin.

2. Conditions for the generation of dityrosine

2.1. Peroxidases

The natural occurrence of dityrosine in examples such as the elastic ligaments of insects (Andersen, 1963, 1964), the ascospore cell walls of *Saccharomyces cerevisiae* (Briza et al., 1986), the cell walls of *Candida albicans* (Smail et al., 1995), the cuticles of *Haemonchus contortus* (Fetterer et al., 1990) and *Ascaris suum* (Fetterer et al., 1993), the cross-linked fertilization envelope of the sea

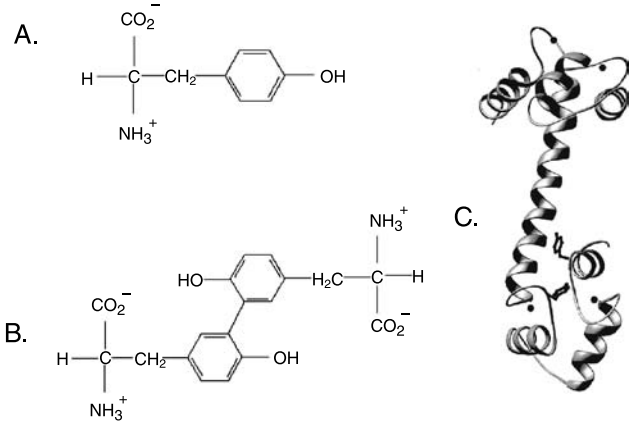

Fig. 1. Structures of the subjects of this review. **A** tyrosine, **B** dityrosine, **C** bovine brain calmodulin. The skeletal outline of the calcium-calmodulin complex shows the positions of the four bound calcium ions (spheres) within calcium-binding domains I and II (N-terminal lobe) and III and IV (C-terminal lobe) and of Tyr-99 and Tyr-138 within the C-terminal lobe. The illustration of calmodulin was provided by William J. Cook

urchin egg *Stronglycocentrotus purpuratus* (Foerder and Shapiro, 1977), the chorion layer of the mature eggs of the mosquito *Aedes aegypti* (Li et al., 1996), prothyroid hormone (Herzog et al., 1992), elastin (LaBella et al., 1967), and collagen (LaBella et al., 1968) suggested that peroxidase-catalyzed tyrosine coupling reactions occur *in vivo*. Ovoperoxidases that catalyze the reaction of H_2O_2 with protein tyrosyl side chains have been isolated from both sea urchin eggs (Deits et al., 1984) and the eggs of the mosquito (Han et al., 2000).

Peroxidases that catalyze reactions leading to tyrosyl radicals and other reactive species have roles in phenomena such as host defenses, inflammation, and the etiology of atherosclerosis. Myeloperoxidase uses the H_2O_2 that is generated by activated neutrophils to yield products that include HOCl from Cl^- and tyrosyl radicals from tyrosine (Heinecke et al., 1993a, b). Four different tyrosyl radical addition products (dityrosine, trityrosine, pulcherosine, and isodityrosine) are synthesized from tyrosine by activated neutrophils (Jacob et al., 1996). The tyrosyl radicals produced in reactions catalyzed by myeloperoxidase catalyze lipid peroxidation, which may be a step in the formation of atherogenic particles (Savenkova et al., 1994; Marquez et al., 1995). Both myeloperoxidase and dityrosine occur in atherosclerotic lesions and plaques (Savenkova et al., 1994; Fu et al., 1998; Kato et al., 2000). Eosinophil peroxidase promotes oxidative tissue injury through the formation of tyrosyl radicals, the production of reactive nitrogen species, and the nitration and bromination of tyrosine (Wu et al., 1999).

The peroxidase-catalyzed cross-linking of tyrosine residues also is a promising tool for protein chemists. Aeschbach et al. (1976) applied horseradish peroxidase to oxidation studies involving fifteen different proteins. In the case of chymotrypsin, the reaction resulted in the average conversion of 85% of the tyrosine residues to dityrosine and total loss of catalytic activity. However, not all proteins react so readily. We tested five commercially available peroxidases for their abilities to catalyze the formation of dityrosine in both random copolymers and several Ca^{2+}-binding proteins (Malencik and Anderson, 1996). All five peroxidases catalyzed dityrosine formation in L-tyrosyl-L-tyrosine, poly (Lys, Tyr) 4:1, and poly (Arg, Tyr) 4:1 but not in poly (Glu, Tyr) 4:1 or poly (Glu, Lys, Tyr) 6:3:1.

Distinct differences among the peroxidases, in both reaction rates and yields, were evident in experiments with poly (Ala, Glu, Lys, Tyr) 6:2:5:1. Listed in their order of effectiveness with this copolymer, the five peroxidases were *Arthromyces* peroxidase > horseradish peroxidase > soybean peroxidase > myeloperoxidase > lactoperoxidase. Of these enzymes, only *Arthromyces* peroxidase catalyzes dityrosine formation in calmodulin, skeletal muscle troponin C, the 20 kD smooth muscle myosin

Table 1. Dityrosyl cross-linked calmodulin derivatives

Derivative	Cross-linking pattern	Conditions of formation
X-CaM[a]	intramolecular Tyr-99 to Tyr-138	UV irradiation (Ca^{2+})
mixed dimers[b]	intermolecular, predominantly Tyr-99 to Tyr-138	UV irradiation (O Ca^{2+}), superoxide dismutase
SP-CaM[c]	[Tyr^8] substance P coupled to Tyr-138	UV irradiation (Ca^{2+}), [Tyr^8] substance P
polymers[d]	intermolecular combinations of Tyr-99 and Tyr-138	*Arthromyces* peroxidase (O Ca^{2+}), H_2O_2

Malencik and Anderson: [a] 1986; [b] 1994; [c] 1987; [d] 1996

light chain, or parvalbumin. The highly polymerized samples of dityrosyl cross-linked calmodulin described in Section 4 were obtained through reactions employing *Arthromyces* peroxidase.

2.2. Other heme proteins: myoglobin, hemoglobin, and cytochrome P450

In addition to the reversible binding of O_2, myoglobin and hemoglobin participate in redox reactions that produce tyrosyl radicals. The perferryl heme moiety that results from the reaction of sperm whale metmyoglobin with H_2O_2 accepts electrons from tyrosine-103, thus generating a tyrosyl radical that participates in radical transfer reactions with other tyrosyl side chains. The major product is a cross-linked dimer of myoglobin in which tyrosine-103 is coupled intermolecularly to tyrosine-151 (Tew and Ortiz de Montellano, 1988). When lactoperoxidase is added to the reaction mixture immediately after quenching by catalase, the products include myoglobin dimers, lactoperoxidase dimers, and myoglobin-lactoperoxidase dimers (Lardinois and Ortiz de Montellano, 2001).

The exposure of pure hemoglobin to a flux of H_2O_2 results in oxidation and minor fragmentation of the protein. The oxidized hemoglobin, either prepared *in vitro* or isolated from oxidatively stressed red blood cells, is selectively degraded by the proteasome (Giulivi and Davies, 1993, 2001). Following both these and other observations, Giulivi and Davies (1993), Heinecke et al. (1993) and Huggins et al. (1993) independently proposed that dityrosine is a useful marker for oxidized proteins.

The perferryl heme that is produced upon the NADPH activation of cytochrome P450 is the key to the oxidation and rapid proteolysis observed in microsomes (Mukhopathy et al., 1994) and the elevation of protein dityrosine that occurs within activated neutrophils (Bhattachargee et al., 2001).

2.3. Ultraviolet irradiation and the influence of superoxide dismutase

Tyrosyl radicals are formed upon the photo-ejection of electrons that accompanies the ultraviolet irradiation of tyrosine (Joschek and Miller, 1966). Lehrer and Fasman (1967) showed that the visible blue fluorescence of poly (L-tyrosine) and copolymers of tyrosine results from the generation of dityrosine during UV irradiation. While becoming acquainted with a new SLM Aminco SPF-500C^{TM} spectrophotofluorometer, we discovered the intramolecular cross-linking of calmodulin tyrosyl residues 99 and 138 (Malencik and Anderson, 1987).

The pattern and extent of photoactivated dityrosine formation in calmodulin are strongly affected by the presence of Ca^{2+} and of superoxide dismutase (SOD) (Fig. 2). The addition of SOD to Ca^{2+}-containing solutions of calmodulin leads to an altered distribution of photoproducts, from a predominance of cross-linked monomer obtained in the presence of Ca^{2+} alone to a mixture exhibiting both inter- and intramolecular cross-linking. When Ca^{2+} is absent, significant dityrosine formation occurs only in the presence of SOD. The resulting reaction mixture yields dimeric calmodulin, a smaller

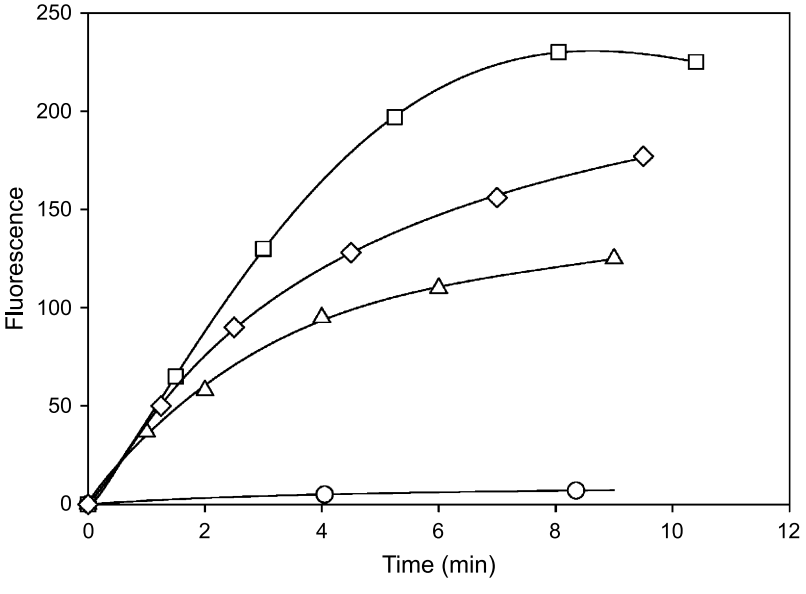

Fig. 2. Effects of superoxide dismutase and varying Ca^{2+} concentration on dityrosine formation occurring during the UV (280 nm) irradiation of calmodulin. The samples contained 15 μM bovine brain calmodulin and ○, 0.25 mM EDTA; △, 0.25 mM EDTA plus 25 μg/ML SOD; □, 0.5 mM $CaCl_2$; or ◇, 0.5 mM $CaCl_2$ plus 25 μg/ML SOD. Fluorescence intensities were measured at 400 nm. (Reprinted with permission from Malencik, D.A. and Anderson, S.R., Biochemistry 33: 13363–13372. Copyright 1994, American Chemical Society)

amount of more highly polymerized material, and virtually no cross-linked monomer (Malencik and Anderson, 1994). Table 1 summarizes the different dityrosyl derivatives of calmodulin that we have isolated from solutions that were UV irradiated under various conditions: i.e. the cross-linked monomer; an adduct containing one mole [Tyr^8] substance P per mole calmodulin, covalently attached to Tyr-138; and a fraction of cross-linked calmodulin *dimers*.

The electrons that are ejected during the ultraviolet irradiation of tyrosine react with molecular oxygen to produce the *superoxide radical anion* $O_2^{\bullet -}$, which in turn participates in reactions with the tyrosyl radical that compete with coupling. The following scheme incorporates reactions outlined by Holler and Hopkins (1989) and additionally includes the formation of tyrosine hydroperoxides (Jin et al., 1993).

$$\text{excitation } \mathrm{TyrOH} \xrightarrow{h\nu} \mathrm{TyrOH}* \tag{1}$$

$$\text{electron ejection } \mathrm{TyrOH}* \rightarrow \mathrm{TyrO}^{\bullet} + \mathrm{H}^{+} + \mathrm{e}^{-} \tag{2}$$

$$\text{superoxide formation } \mathrm{e}^{-} + \mathrm{O}_2 \rightarrow \mathrm{O}_2^{\bullet -} \tag{3}$$

$$\text{coupling } \mathrm{TyrO}^{\bullet} \rightarrow \frac{1}{2}\text{dityrosine} \tag{4}$$

$$\text{repair } \mathrm{O}_2^{\bullet -} + \mathrm{TyrO}^{\bullet} \rightarrow \mathrm{TyrOH} + \mathrm{O}_2 \tag{5}$$

$$\text{hydroperoxide formation } \mathrm{O}_2^{\bullet -} + \mathrm{H}^{+} + \mathrm{TyrO}^{\bullet} \rightarrow \text{Tyrosine hydroperoxides} \tag{6}$$

$$\text{disproportionation } \mathrm{O}_2^{\bullet -} + \mathrm{H}^{+} \xrightarrow[\mathrm{SOD}]{} \tfrac{1}{2}\mathrm{H}_2\mathrm{O}_2 + \tfrac{1}{2}\mathrm{O}_2 \tag{7}$$

Removal of the superoxide radical anion in reaction 7 inhibits both the repair process in reaction 5 and hydroperoxide formation of reaction 6, thus prolonging the lifetime of the tyrosyl radical and increasing the probability that intermolecular coupling will occur. The inclusion of catalase in the reaction media has negligible effects. Superoxide dismutase alone, in either the presence or absence of H_2O_2, catalyzes no dityrosine formation in the samples tested. The addition of ascorbic acid, glutathione, dithiothreitol, dihydroxyphenylalanine, Mn^{+2}, or Cu^{2+} to the solutions prior to UV irradiation strongly inhibits dityrosine formation (Malencik and Anderson, 1987; also unpublished).

Stimulatory effects of superoxide dismutase on the production of dityrosine have been observed during the UV irradiation of tyrosine (Holler and Hopkins, 1989), the riboflavin-sensitized photooxidation of collagen (Kato et al., 1994), the γ-irradiation of polypeptides (Prutz et al., 1983), the *Arthromyces* peroxidase-catalyzed cross-linking of calmodulin (Malencik and Anderson, 1996), and the activation of neutrophils (Jacob et al., 1996).

The yields of dityrosine obtained in the UV-irradiated solutions are inevitably low. The reactions appear to be self-limiting, with substantial amounts of unreacted tyrosine remaining after ~6% conversion to dityrosine in the case of calmodulin. Additional reactions that are not included in the preceding scheme may compete with coupling. For example, some of the tyrosyl radicals may disproportionate to tyrosine and dihydroxyphenylalanine (DOPA). This route would generate a species, DOPA, that completely inhibits the formation of dityrosine when it is added to previously unexposed solutions of calmodulin (unpublished result).

2.4. Reactive nitrogen species: nitric oxide, peroxynitrite, and nitrogen dioxide

Dityrosine and 3-nitrotyrosine may occur simultaneously under conditions of nitrative stress. The potent oxidant peroxynitrite, formed from the reaction of superoxide anion $O_2^{\bullet -}$ with $NO^{\bullet}$, is linked to inflammatory and degenerative diseases (Beckman, 1990). van der Vliet et al. (1994, 1995) showed that the reactions of phenylalanine and tyrosine with peroxynitrite result in the nitration of both amino acids and, in addition, the formation of dityrosine. Tyrosyl radicals, detected in electron paramagnetic measurements (Santos et al., 2000), are produced in the original reaction with peroxynitrite and/or in secondary reactions with the decomposition product NO_2 (Lymar et al., 1996). The tyrosyl radicals take part in competing reactions, notably condensation with other tyrosyl radicals to give dityrosine and reaction with NO_2 to give 3-nitrotyrosine. The ratio of the two products depends on environment (Zhang et al., 2001) and on pH, concentration and reaction rates (Lymar et al., 1996). An example of tyrosine nitration occurs in rejected renal allographs. Manganese superoxide dismutase from rejected tissue contains 3-nitrotyrosine and exhibits diminished catalytic activity (MacMillan-Crow et al., 1996). The *in vitro* reaction of manganese superoxide dismutase with peroxynitrite results in the formation of both 3-nitrotyrosine and dityrosine (MacMillan-Crow et al., 1998).

Peroxynitrite is not the only nitrogen radical that reacts with proteins. NO_2, which is an atmospheric pollutant, reacts with both tryptophan and tyrosine. Dityrosine and 3-nitrotyrosine were detected in proteins that had been

exposed to NO_2 (Kikugawa et al., 1994). The *in vitro* reaction of nitrite with HOCl yields a species similar to nitryl chloride that reacts with N-acetyl-L-tyrosine to produce N-acetylated chlorotyrosine, nitrotyrosine, and dityrosine (Eiserich et al., 1996). These reactions provide mechanisms for tyrosine nitration that are independent of peroxynitrite.

2.5. Reactive oxygen species: the hydroxyl radical

Oxidation of tyrosine by the hydroxyl radical ($^{\bullet}OH$) is less specific than the peroxidase-catalyzed or UV irradiation-induced formation of dityrosine. All amino acids are susceptible to $^{\bullet}OH$, with tyrosine, tryptophan, histidine, and cysteine being the most reactive. Exposure of proteins to ·OH results in major chemical and structural changes. The appearance of 400 nm fluorescence, which was assumed to be indicative of dityrosine formation, showed a linear dose dependence on $^{\bullet}OH$ with apparent inhibition by $O_2^{\bullet-}$ during the γ-irradiation of bovine serum albumin (Davies et al., 1987; Davies and Delsignore, 1987).

Metal-catalyzed oxidation of proteins, carried out in solutions containing 0.1 mM $CuSO_4$ and 1–10 mM H_2O_2, and radiolysis with 18 MV X-rays give differing distributions of products. In the Cu^{2+}-catalyzed oxidations of ribonuclease and lysozyme, the fractions of the resulting 400 nm fluorescence that are due to dityrosine are 100% and 60%, respectively. In contrast, the corresponding fractions obtained from X-ray radiolysis are only 4% and 16% (Huggins et al., 1993). The $CuSO_4$–H_2O_2 system is more effective than Fe EDTA–H_2O_2, Cu^{2+}-ascorbate, or Fe EDTA-ascorbate in the *in vitro* formation of dityrosine in eye lens proteins (Kato et al., 2001). Oxidation products in addition to dityrosine, including carbonyls, o-tyrosine and dihydroxyphenylalanine, are present in proteins that have reacted with $^{\bullet}OH$. In fact, the protein carbonyl content has been used more widely than dityrosine as a marker for protein oxidation (cf review by Chevion et al., 2000).

2.6. Miscellaneous oxidants

Assorted oxidants of varying physiological relevance also stimulate dityrosine production in proteins. Exposure to ozone resulted in crosslinking and dityrosine formation in glucagon, insulin, ribonuclease, and spectrin (Verweij et al., 1982). Reaction of amino acids with lipid hydroperoxides in the presence of metmyoglobin generated dityrosine plus unidentified fluorescent components (Kikugawa et al., 1991a, b). FMN, but not rose bengal, catalyzed the photosensitized intermolecular crosslinking of a model tyrosine copolymer. The reaction required O_2 but did not involve singlet O_2, $O_2^{\bullet-}$, or H_2O_2 (Spikes et al., 1999).

In vitro reaction with hypochlorite results in the crosslinking of low density lipoprotein (LDL) (O'Connell et al., 1994) and of actin (Dalle-Donne et al., 2001). However, the extent of crosslinking of LDL exceeds that expected from the amount of dityrosine that is produced while the fluorescence spectrum of the modified actin, with an emission maximum of 375 to 380 nm, is atypical of dityrosine. The actin sample also showed carbonylation and oxidation of cysteine and methionine.

3. Identification and quantitative determination of dityrosine

3.1. Spectroscopic properties

The singly ionized dityrosine chromophore, in which one of the two phenolic hydroxyl groups is dissociated, is responsible for the 400 nm range emission of dityrosine (Gross and Sizer, 1959; Lehrer and Fasman, 1967). The ionized and unionized forms of dityrosine have absorption maxima at 315 nm and 283 nm, respectively. Either absorption measurements or fluorescence measurements obtained with an excitation wavelength of 320 nm show that the phenolic pK_a of free dityrosine is 7.0 to 7.1 (Lehrer and Fasman, 1967; Malencik and Anderson, 1991). When solutions of dityrosine with $pH \ll 7$ are excited at the absorption maximum of the *unionized* species, 283 nm, the fluorescence emission spectrum of the singly *ionized* form appears. This phenomenon reflects ionization taking place in the excited state, with an apparent $pK_a < 3$.

Figure 3 shows the pH-dependent variations of the excitation spectrum and the near constancy of the emission spectrum of free dityrosine that result from excited state ionization. This phenomenon is characteristic of dityrosine and makes detection possible in solutions with pH values$\ll$7. The data in Table 2 show that the fluorescence emission bands of the dityrosyl calmodulin derivatives are shifted to shorter wavelengths relative to free dityrosine, from 409 nm to 401 nm, and that their ground state pK_a values can be substantially larger than 7. These differences disappear after hydrolysis of the protein.

Borate/boric acid solutions have distinctive effects on the absorption and fluorescence emission spectra of dityrosine (Fig. 4, Table 2). Fluorescence measurements performed as a function of pH and concentration are

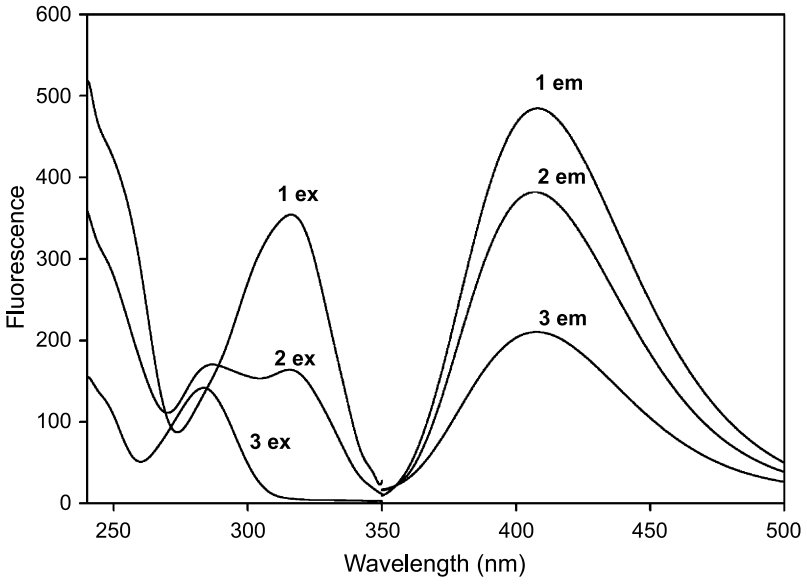

Fig. 3. Ground state ionization of dityrosine demonstrated in pH-dependent fluorescence excitation spectra and excited state ionization demonstrated in pH-independent emission spectra. *1 ex*: pH 9.9, excitation spectrum monitored at a fixed emission λ of 405 nm with slit widths of 3.5 nm. *1 em*: pH 9.9, emission spectrum obtained with a fixed excitation λ of 294 nm with slit widths of 5 nm. *2 ex* and *2 em*: pH 7.0. *3 ex* and *3 em*: pH 2.8. 2.5 μM dityrosine in 0.1 M carbonate, Mops, or acetic acid buffer

Table 2. Fluorescence properties of dityrosine and of dityrosyl crosslinked calmodulin derivatives

Sample	Emission λ_{max} nm	Quantum yield[n]	Excited state lifetime ns	pK_a^h
Dityrosine[a]	409	0.80	4.3 (97%)[f]	7.0–7.1
X-CaM (monomer)[b]				
O Ca^{++}	400	–	4.3 (80%)[f]	7.88
3 mM Ca^{++}	400	–	4.5 (83%)[f]	7.59
CaM dimers[c]				
O Ca^{++}	400	–	–	8.5–8.6
1 mM Ca^{++}	400	–	–	8.5–8.6
CaM polymers[d]				
O Ca^{++}	401	–	4.15 (90%)[g]	8.0–8.1
1 mM Ca^{++}	401	–	4.15 (85%)[g]	8.2–8.3
SP-CaM[e]				
O Ca^{++}	400	–	–	8.8
3 mM Ca^{++}	400	–	–	8.8

Malencik and Anderson: [a] 1992; [b] 1986; [c] 1994; [d] 1996; [e] 1987; [f] Small and Anderson, 1988; [g] Helms et al., 1998; [h] excitation at 320 nm

consistent with a 1:1 complex which dissociates to boric acid and singly ionized dityrosine ($K_1 = 17$ mM) and to monoborate ion and unionized dityrosine ($K_2 = 0.1$ mM) (Malencik and Anderson, 1991). The dityrosyl calmodulins also interact with monoborate/boric acid, but with higher values of K_1 and K_2 that may reflect on the accessibilities of the crosslinks or on limited rotations about the biphenyl bonds.

In certain cases, fluorescence measurements are insensitive to the presence of dityrosine. For example, elevated pK_a values such as that of the cross-linked adduct of [Tyr^8] substance P with calmodulin (Table 2) result in low fluorescence intensities when pH 7 solutions are excited at 320 nm. Fluorescence resonance energy transfer from dityrosine to another chromophore, such as a heme group, also results in decreased sensitivity. The fluorescence of oxidized hemoglobin is detectible only after proteolysis (Giulivi and Davies, 1993).

Dityrosine is a potential energy transfer donor to both ionized and unionized 3-nitrotyrosine. Beginning with the corrected fluorescence emission spectrum of free dityrosine and the absorption spectra of 3-nitrotyrosine, recorded at both high and low pH, we calculated characteristic energy transfer distances (R_o) of 33.6 Å for the dityrosine-ionized 3-nitrotyrosine donor-acceptor pair

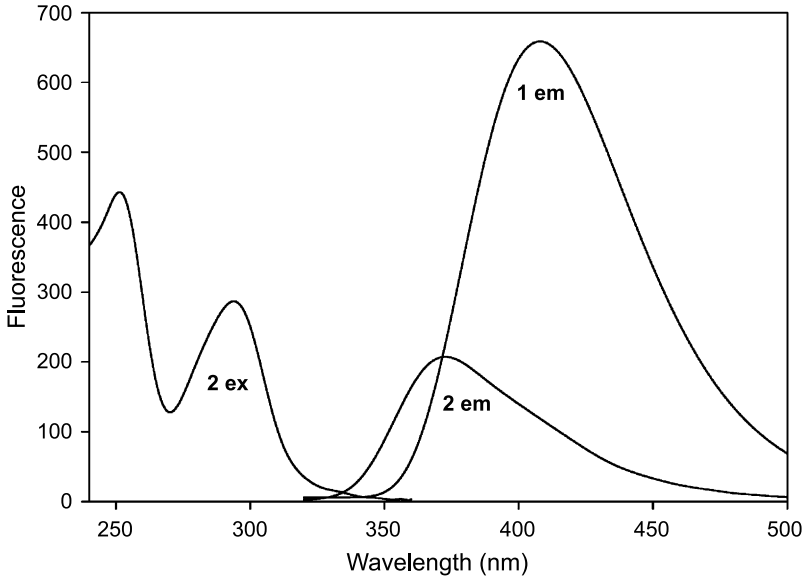

Fig. 4. Fluorescence excitation (*2 ex*) and emission (*2 em*) spectra of dityrosine in presence of 0.5 M boric acid/monoborate, pH 8.3. 1 em shows the pH 9.9 emission spectrum of dityrosine in the absence of boric acid/monoborate. Excitation spectrum: monitored at fixed emission λ of 3.74 nm. Emission spectra: monitored at fixed excitation λ of 304 nm. Slit widths: 5 nm

and 25.6 Å for the dityrosine-unionized 3-nitrotyrosine pair.

The efficiency of energy transfer (E) is critically dependent on the distance (r) between donor and acceptor (cf Lakowicz, 1993).

$$E = R_o^6/\left(R_o^6 + r^6\right)$$

The simultaneous occurrence of dityrosine and 3-nitrotyrosine in the same protein molecule may result in energy transfer and quenching, depending on the value of r. If $r < R_o$, dityrosine could escape detection in fluorometric monitoring.

Table 3 summarizes extinction coefficients that were independently determined in two laboratories (Malencik et al., 1996; Jacob et al., 1996) for standardized solutions of dityrosine. These values are useful for the determination of concentrations from absorption measurements and for the preparation of dityrosine standards.

Table 3. Near ultraviolet absorption properties of free dityrosine

Conditions	λ_{max} (nm)	ε ($cm^{-1}\,M^{-1}$)
carbonate or phosphate, pH 9.9	315	8,380[a]
0.1 M NH_4OH	315	8,600[b]
phosphoric or acetic acid, pH 4.0	283	5,680[a]
0.1 M HCl	283	5,400[b]
0.5 M potassium borate, boric acid, pH 8.0	294	7,700[a,c]

[a] Malencik et al., 1996. Concentrations were based on elemental analysis and determination of the water content of the purified dityrosine preparation; [b] Jacob et al., 1996. Concentrations were determined by scintillation counting of ^{14}C-labeled dityrosine; [c] Malencik and Anderson, 1992

3.2. Chromatographic identification of dityrosine in protein hydrolysates

Fluorescence emission near 400 nm is not necessarily indicative of dityrosine. For example, in the case of lens proteins from subjects of all ages, less than 1% of the dityrosine-like fluorescence is due to dityrosine (Wells-Knecht et al., 1993). However, quantitative determination of dityrosine is possible with a combination of spectroscopic and chromatographic analyses.

Current analyses for dityrosine most often employ reversed phase HPLC with either fluorometric (Malencik et al., 1996; Huggins et al., 1993; Giulivi and Davies, 1993) or electrochemical (Hensley et al., 1998; Wu et al., 1999; Pfeiffer et al., 2000) detection or gas chromatography in conjunction with mass spectrometry (Wells-Knecht et al., 1993; Jacob et al., 1996).

Figure 5 shows the isocratic reversed-phase HPLC analysis of a 0.8 nmol sample of dityrosine from an unfractionated *Arthromyces* peroxidase–H_2O_2– oxidized calmodulin preparation, with superimposed profiles that were obtained with standards consisting of 2 nmol dityrosine and 19 nmol tyrosine (Malencik et al., 1996). Dityrosine accounted for all of the 400 nm fluorescence of this sample, which originally contained 0.72 mol dityrosine/mol protein. Fast atom bombardment mass spectrometry confirmed the expected mass of dityrosine.

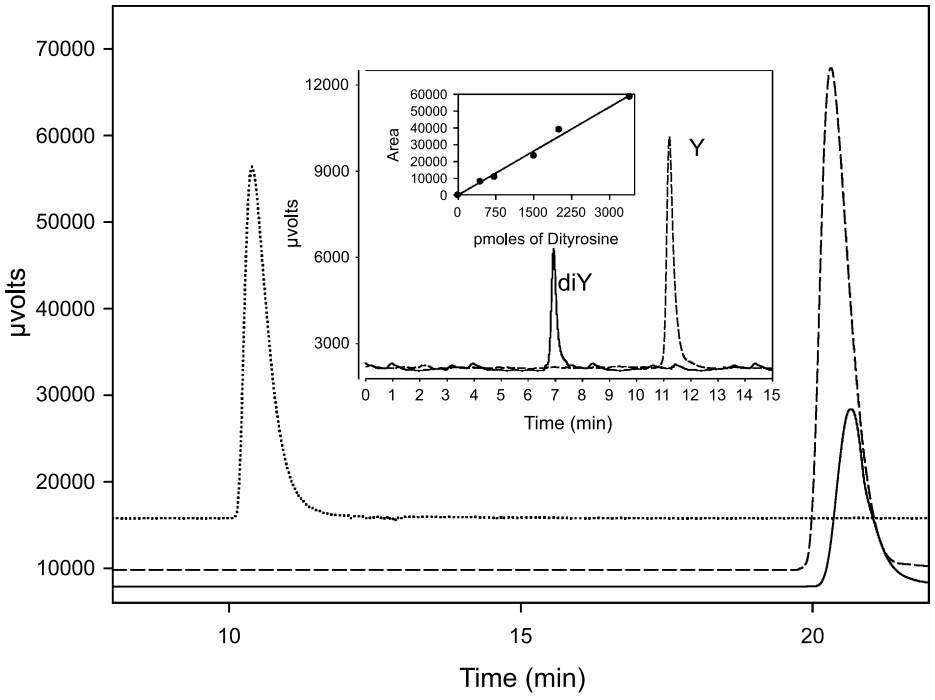

Fig. 5. Isocratic reversed-phase HPLC analysis of dityrosine. Separations are shown for 0.8 nmol dityrosine from an acid hydrolysate of enzymatically cross-linked calmodulin (–), 2.0 nmol dityrosine standard (– – –), and 19 nmol tyrosine standard (——). Monitoring with 280 nm excitation and 305 nm or 400 nm emission. Column: ODSII Spherisorb from LC-Resources. Solvent: 92% H_2O, 8% acetonitrile, and 0.1% trifluoroacetic acid. The inset shows reversed phase analysis of *dabsylated* sample of dityrosine from the acid hydrolysate of calmodulin

Our laboratory devised two novel chromatographic methods for the isolation and analysis of dityrosine: affinity chromatography on immobilized phenyl boronate (Matrex Gel PBA-60), which is based on the association of dityrosine with boric acid/monoborate, and *two-dimensional* chromatography on BioGel P-2 (Malencik et al., 1996). In the first step of BioGel P-2 chromatography, application of a protein hydrolysate to a column equilibrated in 0.10 m NH_4HCO_3 results in separation by size. However, the use of a column equilibrated in 0.1 M HCOOH (pH < 3) in the second step leads to reversible adsorption of dityrosine to the matrix. Performance of the two steps completely separates dityrosine from all of the other amino acids normally present in a hydrolysate. pH-dependent chromatography on BioGel P-2 has the advantages of simplicity and applicability to both large preparative scale and small quantities of dityrosine.

3.3. Amino acid analysis by HPLC: determination of 23 amino acids including dityrosine and 3-nitrotyrosine on a single column

Total amino acid analysis relates dityrosine to the overall composition of a protein hydrolysate. We developed a single column reversed-phase HPLC method that resolves the pre-column-reacted derivatives of dityrosine, 3-nitrotyrosine, three phosphorylated amino acids, and 18 other amino acids with 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride) (Malencik et al., 1990). Adjustments to the procedure described by Knecht and Chang (1986) for the reversed-phase HPLC analysis of dabsylated amino acids allows us to determine the five additional amino acids. The resolution shown in Fig. 6 was made possible through the use of a highly substituted Phenomenex Ultracarb 30 column in combination with a basic pH 8.1 gradient. The gradient program, which is described in detail by Malencik et al. (1990), utilizes 20 mM sodium bicarbonate or ammonium bicarbonate, 4% dimethylformamide, pH 8.1 (solvent A) and 90% acetonitrile and 10% 2-propanol (solvent B). With our equipment, the lower limit for accurate detection is near 1 pmol. However, higher sensitivity should be possible with microbore HPLC columns.

Dabsylation facilitates the accurate analysis of all amino acids since the monodabsylated amino acids are roughly equivalent in response, as are the didabsylated amino acids. We also have employed o-phthaldehyde

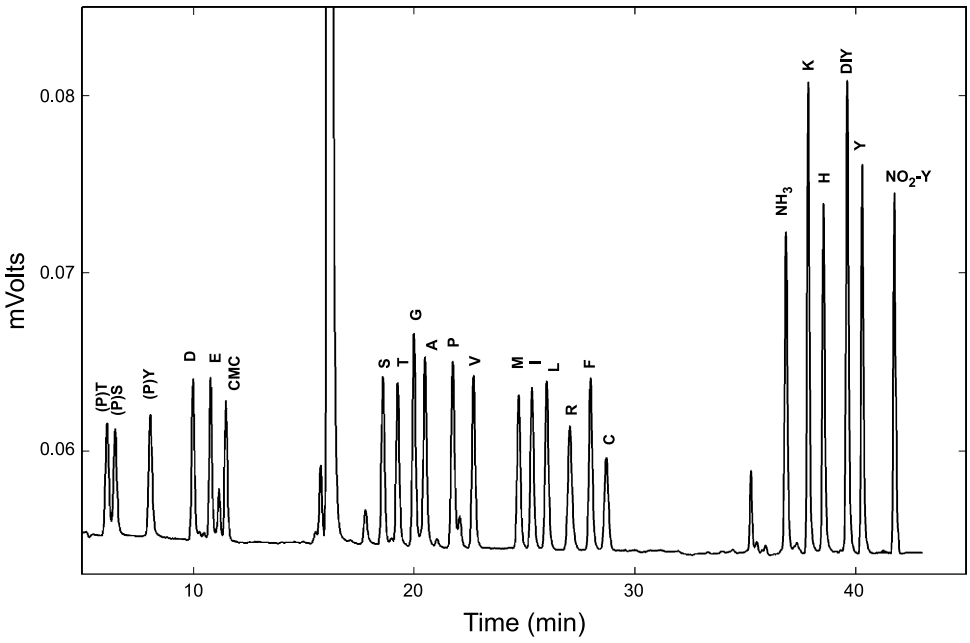

Fig. 6. Separation of 23 dabsylated amino acid standards by reversed phase HPLC on a Phenomenex Ultracarb 30 column using a pH 8.1 gradient. The abbreviations use standard nomenclature except for *DIY*, dityrosine; *NO_2-Y*, 3-nitrotyrosine; *CMC*, carboxymethyl cysteine; *(P)S*, phosphoserine; *(P)T*, phosphothreonine; and *(P)Y*, phosphotyrosine

and dansyl chloride in pre-column derivatization. However, neither approach is as successful as dabsylation. Derivatives obtained with o-phthaldehyde are unstable while the dansylated derivatives vary widely in their individual responses.

Strict cleaning of the hydrolysis tubes with concentrated nitric acid, followed by pyrolysis; hydrolysis of the protein with vapor phase HCl, carried out in a vacuum desiccator; and dabsylation at pH 8.5 instead of the usual pH 9.0 are necessary for optimum results in these analyses.

4. Inter- and intramolecular cross-linking of calmodulin tyrosyl residues

4.1. Structural background

More is known about dityrosine formation in calmodulin than in any other protein. Calmodulin is a good subject for the study of protein dityrosine in light of its role in signal transduction, the fact that it contains just two tyrosyl residues with no cysteine or tryptophan, and the availability of crystallographic data showing the three-dimensional structure of the calcium-calmodulin complex (Babu et al., 1985, 1988; Chattopadhyaya et al., 1992).

The two tyrosyl residues of calmodulin occur within calcium binding sequences (Fig. 1). Tyr-99 faces into the third calcium binding site and is partially accessible to solvent while Tyr-138 points away from the fourth calcium binding site and into a hydrophobic pocket (Babu et al., 1985, 1988). Both the X-ray data and fluorescence resonance energy transfer experiments with an engineered calmodulin (Steiner et al., 1991) show that the average distance between the two tyrosyl side chains is $\sim$15 Å. This perspective raises questions as to whether two tyrosyl radicals within such an arrangement are capable of coupling and, if they are, how the resulting dityrosine crosslink affects the properties of calmodulin.

Time-resolved fluorescence spectroscopy expands our view of calmodulin by showing that calcium binding domains III and IV have significant mutual mobility. Frequency domain anisotropy measurements on the intrinsic tyrosine fluorescence demonstrate fast local rotations, in the subnanosecond range, that are much shorter than the 10 nsec global rotational correlation time of calcium-calmodulin (Gryczynski et al., 1991; Steiner et al., 1988; reviewed by Anderson, 1991). These fast segmental rotations may allow the two side chains to come within reacting distance at some point during the lifetimes of the tyrosyl radicals.

4.2. Cross-linked calmodulin monomer (X-CaM)

The formation of the cross-linked monomer demonstrates that the two tyrosyl side chains of calmodulin indeed approach each other during their excursions. Contact and coupling take place detectibly only in the Ca^{2+}-calmodulin complex. Little photoactivated dityrosine formation occurs in Ca^{2+}-free calmodulin unless superoxide dismutase is present. In that case, the result is intermolecular cross-linking (Section 2.3).

The interactions of the cross-linked monomer with both Ca^{2+} and proteins are weakened but not abolished. Diminished binding of X-CaM to the affinity medium phenylagarose facilitated its separation from both unreacted calmodulin and non-binding by-products. X-CaM fails to activate either calcineurin or smooth muscle myosin light chain kinase in standard catalytic assays unless large excesses, which may include traces of native-like calmodulin, are added (Table 4, Malencik and Anderson, 1987).

Fluorescence anisotropy measurements using the intrinsic fluorescence of dityrosine are sensitive to the conformational changes induced by Ca^{2+} binding and to the association of the cross-linked monomer with enzymes. The 4 ns range of the lifetime of the excited state of dityrosine (Table 2) is ideally suited for observations on the global rotations of proteins the size of calmodulin and on the fast rotations that may occur within protein molecules of any size. Steady state, pulse, and frequency domain fluorescence anisotropy studies showed that conformational changes, consisting of elongation and decreased segmental mobility, accompany the addition of Ca^{2+} to X-CaM (Malencik and Anderson, 1987; Small and Anderson, 1988; Helms et al., 1998). However, the concentrations of Ca^{2+} that are required to produce the changes are much larger than those that produce spectral changes in native calmodulin: i.e.10 mM or greater versus 0.1 mM or less (Table 4).

The fluorescence anisotropy of X-CaM increases when skeletal or smooth muscle myosin light chain kinase, phosphorylase kinase, or calcineurin is added to calcium (5 mM)-containing solutions. Back titrations of the complexes with native calmodulin result in direct displacement of the cross-linked monomer. The competitive displacement of X-CaM from smooth muscle myosin kinase indicates that the dissociation constant of the complex is ~280 fold larger than the dissociation constant of the native calmodulin-enzyme complex.

Within the error of the amino acid analyses, the amino acid composition of X-CaM is the same as that of the native calmodulin control, except for tyrosine and dityrosine.

Table 4. Interactions of dityrosyl calmodulin derivatives with calcium, smooth muscle myosin light chain kinase, and calcineurin

Sample	$[Ca^{2+}]^a_{1/2}$ μM	Enzyme activation	
		MLCK μM NADH/min	Calcineurin activity ratio ± CaM
Untreated[b,c] control CaM	–	− 1.15	2.6
Monomer	~300 (heterogeneous)	0	1.0
Dimers[b,c]	11	− 1.13	2.5
Polymers[c]	~20 (heterogeneous)	− 0.55 to − 0.68	0.8–0.83
SP-CaM[d]	10	− 0.89	–

[a] Concentration of calcium required for a half maximum increase in dityrosine fluorescence anisotropy. Malencik and Anderson: [b] 1994; [c] 1996; [d] 1987

4.3. Adduct of calmodulin with [Tyr^8] substance P (SP-CaM)

The purpose of this experiment was to determine whether a small calmodulin-binding peptide could be coupled to calmodulin (Malencik and Anderson, 1988). We chose [Tyr^8] substance P (ArgProLysProGlnGlnPheTyrGlyLeuMetNH_2) because it is commercially available and contains tyrosine but not tryptophan. Substance P is one of a number of small basic biologically active peptides that we found to undergo Ca^{2+}-dependent binding by calmodulin (Malencik and Anderson, 1982; review by Anderson and Malencik, 1986). The presence of [Tyr^8] substance P, at a ratio of 1.2 mol peptide/mol calmodulin, during the ultraviolet irradiation of calmodulin has a profound effect on the reaction. In fact, no calmodulin derivatives with the properties of the cross-linked monomer X-CaM are detectible in the dityrosine-containing photoproducts. However, the reaction remains strongly Ca^{2+}-dependent.

The conjugate containing [Tyr^8] substance P coupled to calmodulin Tyr-138 (SP-CaM) was purified from the reaction mixture through a series of chromatographic steps. The presence of the coupled peptide was verified by automated sequencing. The gap in the resulting sequence (ArgProLysProGlnGlnPhe–GlyLeuMet) is consistent with the coupling of Tyr-8 to calmodulin, which is not degraded by the Edman reagent. Analyses of the thrombic fragments that were prepared from SP-CaM showed that

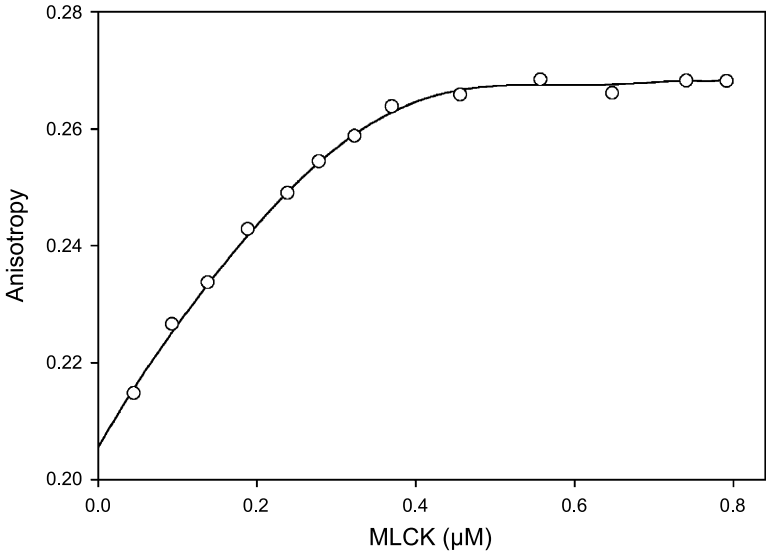

Fig. 7. Fluorescence anisotropy titration of the calmodulin-[Tyr^8] substance P adduct with smooth muscle myosin light chain kinase. Conditions: 0.15 M NaCl, 50 mM Tris, 1 mM $CaCl_2$, pH 8.6 (25°). Excitation λ: 320 nm. Emission: 380 nm cutoff filter. (Reprinted with permission from Malencik, D.A. and Anderson, S.R., Biochemistry 27: 944–950. Copyright 1987, American Chemical Society)

the bound peptide and the dityrosine are associated with calmodulin sequence 107–148.

The functional properties of SP-CaM are close to those of native calmodulin. Coupled catalytic assays, in which NADH oxidation is continuously monitored in a medium that includes 10 nM smooth muscle myosin light chain kinase, 10 nM calmodulin or derivative, 0.2 mM Ca^{2+}, show that under these conditions SP-CaM is 77% as effective as the unmodified control (Table 4).

Figure 7 shows a binding experiment that employs the dityrosine fluorescence of SP-CaM. The stepwise addition of smooth muscle myosin light chain kinase to a solution containing 0.4 to 0.5 μM SP-CaM results in increases in fluorescence anisotropy up to the end point of ~1 mol MLCK/mol SP-CaM. No further changes in anisotropy occur at higher concentrations of the enzyme. The levels of native calmodulin that are required to displace SP-CaM indicate that the dissociation constant of SP-CaM-MLCK is ~56–83% of the value (1.8 nM) for native CaM-MLCK (Malencik and Anderson, 1986). The addition of varying concentrations of Ca^{2+} to Ca^{2+}-free SP-CaM also results in increasing anisotropies, with a midpoint of 10 μM Ca^{2+} that corresponds to the average K_d for Ca^{2+}-native calmodulin complexes (Crouch and Klee, 1980). The concentration dependence is similar to that illustrated in the next section for the cross-linked dimers (Fig. 8).

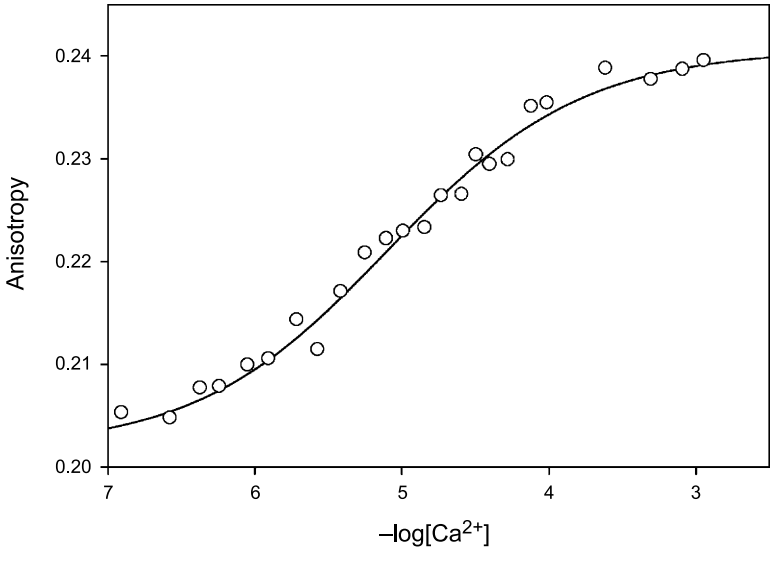

Fig. 8. Fluorescence anisotropy titration of the calmodulin dimers as a function of total $CaCl_2$ concentration (M). Conditions: 13 μg/ML protein in 0.15 M NaCl, 50 M Tris, pH 8.6 (25°). Excitation: 320 nm. Emission: 400 nm. (Bandwidths were 5 and 10 nm, respectively.) (Reprinted with permission from Malencik, D.A. and Anderson, S.R., Biochemistry 33: 13363–13372. Copyright 1994, American Chemical Society)

4.4. Cross-linked calmodulin dimers

The dimers that form during the ultraviolet irradiation of Ca^{2+}-free calmodulin in the presence of superoxide dismutase closely resemble native calmodulin in their interactions (Table 4). The purified dimers correspond to a single component of molecular weight $33{,}750 \pm 180$ in sedimentation equilibrium, which is not affected by molecular shape, and to three components in $NaDodSO_4$ polyacrylamide gel electrophoresis experiments, which reflect both molecular weight and Stoke's radius. The predominant cross-link in the dimers is intermolecular, connecting Tyr-99 to Tyr-138 (Malencik and Anderson, 1994). Lesser amounts of the Tyr-99 to Tyr-99 crosslinked dimer are also present.

The behavior of the cross-linked dimers in catalytic assays of smooth muscle myosin light chain kinase is indistinguishable from that of unmodified calmodulin. Both cases give the same values for V_{max} and K_m, with $K_m = 1$ nM calmodulin (expressed in terms of the monomer unit). Fluorescence titrations with a myosin light chain kinase probe give binding ratios of 1 mol MLCK/mol native calmodulin and 1.7 mol MLCK/mol dimer.

Figure 8 shows the gradual increases in fluorescence anisotropy that accompany the incremental addition of Ca^{2+} to a solution of the purified dimers. Since the fluorescence intensity of the sample is nearly independent of Ca^{2+}, the results are easily fit by a two-component analysis that is based on the principle of addivity of anisotropies (Weber, 1952). The analysis assumes that virtually all of the Ca^{2+} is free and that φ, the average saturation of the protein with Ca^{2+}, can be calculated from the observed anisotropy (A_{obs}) and the anisotropies of the Ca^{2+}-free dimers (A_o) and the Ca^{2+}-saturated dimers (A_c):

$$\varphi = (A_{obs} - A_o)/(A_c - A_o)$$

The smooth curve in Fig. 8 was calculated for $A_o = 0.202$, $A_c = .241$, n (Hill coefficient) $= 0.727$, $C_{1/2}$ (concentration of Ca^{2+} when $\varphi = 0.5) = 12.3\ \mu$M. Subtraction of the estimated concentration of bound Ca^{2+} gives $C_{1/2} = 11\ \mu$M free Ca^{2+}, which is close to the average dissociation for the native calmodulin-Ca^{2+} complex determined at pH 7.5 by equilibrium dialysis (Crouch and Klee, 1980).

A credible model for the complex of two myosin light chain kinase molecules with a cross-linked calmodulin dimer would assume the flexibility of the intermolecular cross-link, as pursued in the next section. It would consider that the enzyme's calmodulin-binding domain (approximately residues 796 to 815) is located near its C-terminal end (residue 972) (Olson et al., 1990) and that the enzyme is an elongated molecule with the hydrodynamic properties of an ellipsoid measuring $26\ \text{Å} \times 500\ \text{Å}$ (Ausio et al., 1992). Following the results of X-ray crystallographic studies on a peptide-calmodulin model (Meador et al., 1992), the N- and C-terminal domains of each of the two cross-linked calmodulin molecules would be cupped around a segment of enzyme within residues 796–815. The side chains of Tyr-99 and Tyr-138 would not be in contact with the enzyme.

4.5. Enzymatically polymerized calmodulin

Achievement of our goal of increased cross-linking efficiency through the replacement of the photochemical reaction with *Arthromyces* peroxidase-catalyzed coupling also resulted in higher degrees of polymerization that are dependent on the concentration of calmodulin. For maximum effects, we emphasized the preparation of high molecular weight calmodulin samples. Establishment of optimum reaction conditions included fluorometric monitoring of time courses, the use of minimum quantities of H_2O_2, and quenching of the reactions by addition of glutathione and glutathione peroxidase (Malencik and Anderson, 1996). Following gel filtration and affinity chromatography, we recovered ~40% of the initial calmodulin as polymers that contain close to the maximum possible amount of dityrosine. Although less highly polymerized fractions show discrete bands on $NaDodSO_4$ polyacrylamide gel electrophoresis, the material isolated from a reaction carried out at 50 mg/mL calmodulin appears as a continuum of species having higher molecular weights than the 96 kD glycogen phosphorylase standard (Fig. 9).

Analytical ultracentrifugation demonstrates both the association of the calmodulin polymers with smooth muscle myosin light chain kinase and the dissociation that follows the addition of either EDTA or the high affinity calmodulin-binding peptide mastoparan (Malencik and Anderson, 1983). As presaged by the behavior of the calmodulin dimers, the highly polymerized preparation retains a significant proportion (50–60%) of the activity of untreated calmodulin in the myosin light chain kinase assay (Table 4). The polymers do not activate calcineurin, but instead slightly inhibit the enzyme in assays with p-nitrophenyl phosphate (Table 4).

Frequency domain fluorescence anisotropy measurements on the high molecular weight polymers give peak rotational correlation times of 0.6 ns (zero Ca^{2+}) and

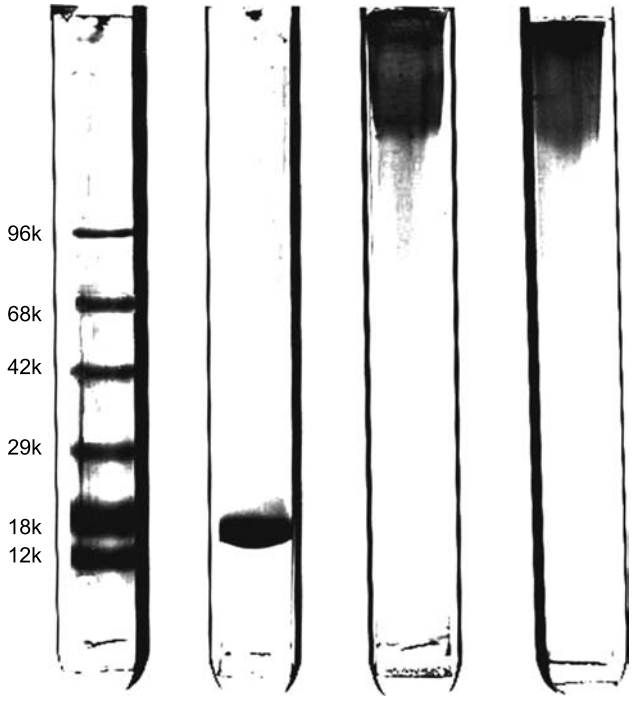

Fig. 9. $NaDodSO_4$ polyacrylamide gel (7.5%) electrophoresis performed (from left to right) on standards, unmodified calmodulin control, and the most highly polymerized fractions containing 0.98 mol dityrosine/mol monomer unit

1.2 ns ($+Ca^{2+}$), showing that the intermolecularly cross-linked segments of the polymers possess a degree of the mobility that is characteristic of the tyrosine-containing sequences of native calmodulin (Helms et al., 1998). As the result of their ability to reorient, the individual units of a flexibly cross-linked polymer could be more accessible to an enzyme such as myosin light chain kinase than the units of a rigidly cross-linked polymer.

The ability of calmodulin to participate in these reactions together with the properties of the cross-linked dimers and higher molecular weight species provide a view of calmodulin as a mobile, accommodating molecule.

5. Applications of the phenolic coupling of protein tyrosyl residues

Most protein cross-linking procedures target exposed amino acid side chains such as asp, glu, lys, and cys. Diazonium conjugation, which is irreproducible and inefficient, is the only method other than phenolic coupling that is available for the cross-linking of tyrosyl side chains (Hermanson, 1996). Our success in the cross-linking of calmodulin suggests the use of peroxidase catalyzed cross-linking in the preparation of protein conjugates and polymers. The irreversible cross-linking of protein molecules with dityrosine, a naturally occurring amino acid, should be useful in peptide and protein drug design. Tyrosine residues could be placed within suitable proximities for enzymatically cross-linked engineered proteins.

Tyrosine cross-linking is a major component of the art of dough mixing and breadmaking. Glutenin subunits contain 3–5% tyrosine, with tyrosine residues often occurring in repeats of TyrTyr pairs through the sequence. The conversion of glutenin to gluten is accompanied by interchain dityrosine formation that is facilitated by natural enzymatic activity and by the potassium bromate that is added to flour (Tilley et al., 2001). Coupling of proteins and peptides with either tyrosine or derivatives of tyrosine such as tyramine may be useful for fluorescent labeling. Larios et al. (2001) employed a fluorophore-labeled tyramide as a probe to detect tyrosyl radicals in fibroblast-derived extracellular matrix proteins.

Acknowledgment

We thank William J. Cook for preparing the illustration of calmodulin.

Our work on dityrosine has been supported by the National Institutes of Health, which has provided us with 24 years of funding through DDK13912; the Medical Research Foundation of Oregon; and the Oregon State University Foundation.

References

Aeschbach R, Amado R, Neukom H (1976) Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues. Biochim Biophys Acta 439: 292–301

Andersen SO (1963) Characterization of a new type of cross-linkage in resilin, a rubber-like protein. Biochim Biophys Acta 69: 249–262

Andersen SO (1964) The cross-links in resilin identified as dityrosine and trityrosine. Biochim Biophys Acta 93: 213–215

Anderson SR (1991) Time-resolved fluorescence spectroscopy: applications to calmodulin. J Biol Chem 266: 11405–11408

Anderson SR, Malencik DA (1986) Peptides recognizing calmodulin. Calcium and Cell Function (Academic Press) 7: 1–42

Ausio JA, Malencik DA, Anderson SR (1992) Analytical sedimentation studies of turkey gizzard myosin light chain kinase and telokin. Biophys J 61: 1656–1663

Babu YS, Sacks JS, Greenhough TG, Bugg CE, Means AR, Cook WJ (1985) Three dimensional structure of calmodulin. Nature 315: 37–40

Babu YS, Bugg CE, Cook WJ (1988) Structure of calmodulin refined at 2.2 Å resolution. J Mol Biol 204: 191–204

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman B (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624

Bhattacharjee S, Pennathur S, Byun J, Crowley J, Mueller D, Gischler J, Hotchkiss RS, Heinecke JW (2001) NADPH oxidase of neutrophils elevates o,o′-dityrosine cross-links in proteins and urine during inflammation. Arch Biochem Biophys 395: 69–77

Briza P, Winkler G, Kalchhauser H, Breitenbach M (1986) Dirytosine is a prominent component of the yeast ascospore wall. A proof of its structure. J Biol Chem 261: 4288–4294

Chattopadhyaya R, Meador WE, Means AR, Quiocho FA (1992) Calmodulin structure refined at 1.7 Å resolution. J Mol Biol 228: 1177–1192

Chevion M, Berenshtein E, Stadtman ER (2000) Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radic Res 33 [Suppl]: S99–S108

Crouch TH, Klee CB (1980) Positive cooperative binding of calcium to bovine brain calmodulin. Biochemistry 19: 3692–3698

Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, Di Simplicio P, Colombo R (2001) Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. Free Radic Biol Med 31: 1075–1083

Davies KJ, Delsignore ME (1987) Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. J Biol Chem 262: 9908–9913

Davies KJ, Delsignore ME, Lin SW (1987) Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J Biol Chem 262: 9902–9907

Deits T, Farrance M, Kay ES, Medill L, Turner EE, Weidman PJ, Shapiro BM (1984) Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchin egg. J Biol Chem 259: 13525–13533

Eiserich JP, Cross CE, Jones AD, Halliwell B, van der Vliet A (1996) Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. J Biol Chem 271: 19199–19208

Fetterer RH, Rhoads ML (1993) Biochemistry of the nematode cuticle relevance to parasitic nematodes of livestock. Vet Parasitol 46: 103–111

Fetterer RH, Rhoads ML, Urban JF Jr (1993) Synthesis of tyrosine-derived cross-links in *Ascaris suum* cuticular proteins. J Parasitol 79: 160–166

Foerder CA, Shapiro BM (1977) Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine cross-links. Proc Natl Acad Sci 74: 4214–4218

Fu S, Davies MJ, Stocker R, Dean RI (1998) Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem J 333 (Pt 3): 519–525

Giulivi C, Davies KJ (1993) Dityrosine and tyrosine oxidation products are endogenous markers for the selective proteolysis of oxidatively modified red blood cell hemoglobin by (the 19 S) proteasome. J Biol Chem 268: 8752–8759

Giulivi C, Davies KJ (2001) Mechanism of the formation and proteolytic release of H_2O_2-induced dityrosine and tyrosine oxidation products in hemoglobin and red blood cells. J Biol Chem 276: 24129–24136

Gross AJ, Sizer JW (1959) The oxidation of tyramine, tyrosine, and related compounds by peroxidase. J Biol Chem 234: 1611–1614

Gryczynski I, Lakowicz JR, Steiner RF (1988) Frequency-domain measurements of the rotational dynamics of the tyrosine groups of calmodulin. Biophys Chem 30: 49–59

Han Q, Li G, Li J (2000) Purification and characterization of chorion peroxidase from *Aedes aegypti* eggs. Arch Biochem Biophys 378: 107–115

Heinecke JW, Li W, Daehnke HL 3rd, Goldstein JA (1993a) Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages. J Biol Chem 268: 4069–4077

Heinecke JW, Li W, Francis GA, Goldstein JA (1993b) Tyrosyl radical generated by myeloperoxidase catalyzes the oxidative cross-linking of proteins. J Clin Invest 91: 2866–2872

Helms MK, Malencik DA, Anderson SR (1998) Flexibility involving the intermolecular dityrosyl cross-links of enzymatically polymerized calmodulin. Biochemistry 37: 8378–8384

Hensley K, Maidt ML, Yu Z, Sang H, Markesbery WR, Floyd RA (1998) Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J Neurosci 18: 8126–8132

Hermanson GT (1996) Bioconjugate techniques. Academic Press, New York, pp 419–455

Herzog V, Berndorfer U, Saber Y (1992) Isolation of insoluble secretory product from bovine thyroid: extracellular storage of thyroglobulin in covalently cross-linked form. J Cell Biol 118: 1071–1083

Holler TP, Hopkins PB (1989) A qualitative fluorescence-based assay for tyrosyl radical scavenging activity: ovothiol A is an efficient scavenger. Anal Biochem 180: 326–330

Huggins TG, Wells-Knecht MC, Detorie NA, Baynes JW, Thorpe SR (1993) Formation of o-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. J Biol Chem 268: 12341–12347

Jacob JS, Cistola DP, Hsu FF, Muzaffar S, Mueller DM, Hazen SL, Heinecke JW (1996) Human phagocytes employ the myeloperoxidase-hydrogen peroxide system to synthesize dityrosine, trityrosine, pulcherosine, and isodityrosine by a tyrosyl radical-dependent pathway. J Biol Chem 271: 19950–19956

Jin F, Leitich J, von Sonntag C (1993) The superoxide radical reacts with tyrosine-derived phenoxyl radicals by addition rather than by electron transfer. J Chem Soc Perkin Trans 2: 1583–1586

Joschek HI, Miller SI (1966) Photooxidation of phenol, cresols, and dihydroxybenzenes. J Am Chem Soc 88: 3273–3281

Kato Y, Wu X, Naito M, Nomura H, Kitamoto N, Osawa T (2000) Immunochemical detection of protein dityrosine in atherosclerotic lesion of apo-E-deficient mice using a novel monoclonal antibody. Biochem Biophys Res Commun 275: 11–15

Kato Y, Kitamoto N, Kawai Y, Osawa T (2001) The hydrogen peroxide/copper ion system, but not other metal-catalyzed oxidation systems, produces protein-bound dityrosine. Free Radic Biol Med 31: 624–632

Kikugawa K, Kato T, Hayasaka A (1991a) Formation of dityrosine and other fluorescent amino acids by reaction of amino acids with lipid hydroperoxides. Lipids 26: 922–929

Kikugawa K, Kato T, Beppu M, Hayasaka A (1991b) Development of fluorescence and cross-links in eye lens crystallin by interaction with lipid peroxy radicals. Biochim Biophys Acta 1096: 108–114

Kikugawa K, Kato T, Okamoto Y (1994) Damage of amino acids and proteins induced by nitrogen dioxide, a free radical toxin, in air. Free Radic Biol Med 16: 373–382

Knecht R, Chang JY (1986) Liquid chromatographic determination of amino acids after gas-phase hydrolysis and derivatization with (dimethylamino) azobenzenesulfonyl chloride. Anal Chem 58: 2375–2379

LaBella F, Keeley F, Vivian S, Thornhill D (1967) Evidence for dityrosine in elastin. Biochem Biophys Res Commun 26: 748–753

LaBella F, Waykole P, Queen G (1968) Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. Biochem Biophys Res Commun 30: 333–338

Lakowicz JR (1983) Energy transfer. In: Lakowicz JR (ed) Principles of fluorescence spectroscopy. Plenum Press, New York, pp 305–337

Lardinois OM, de Montellano PR (2001) H_2O_2-mediated cross-linking between lactoperoxidase and myoglobin: elucidation of protein-protein radical transfer reactions. J Biol Chem 276: 23186–23191

Larios JM, Budhiraja R, Fanburg BL, Thannickal VJ (2001) Oxidative protein cross-linking reactions involving L-tyrosine in transforming growth factor-beta1-stimulated fibroblasts. J Biol Chem 276: 17437–17441

Lehrer SS, Fasman GD (1967) Ultraviolet irradiation effects in poly-L-tyrosine and model compounds. Identification of bityrosine as a photoproduct. Biochemistry 6: 757–767

Li J, Hodgeman BA, Christensen BM (1996) Involvement of peroxidase in chorion hardening in *Aedes aegypti*. Insect Biochem Mol Biol 26: 309–317

Lymar SV, Jiang Q, Hurst JK (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. Biochemistry 35: 7855–7861

MacMillan-Crow IA, Crow JP, Kerby JD, Beckman JS, Thompson JA (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc Natl Acad Sci USA 93: 11853–11858

MacMillan-Crow LA, Crow JP, Thompson JA (1998) Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 37: 1613–1622

Malencik DA, Anderson SR (1982) Binding of simple peptides, hormones, and neurotransmitters by calmodulin. Biochemistry 21: 3480–3486

Malencik DA, Anderson SR (1983) High affinity binding of the mastoparans by calmodulin. Biochem Biophys Res Comm 114: 50–56

Malencik DA, Anderson SR (1986) Calmodulin-linked equilibria in smooth muscle myosin light chain kinase. Biochemistry 25: 709–721

Malencik DA, Anderson SR (1987) Dityrosine formation in calmodulin. Biochemistry 26: 695–704

Malencik DA, Anderson SR (1988) Peptide cross-linking to calmodulin: attachment of [Tyr8]substance P. Biochemistry 27: 944–950

Malencik DA, Anderson SR (1991) Fluorometric characterization of dityrosine: complex formation with boric acid and borate ion. Biochem Biophys Res Commun 178: 60–67

Malencik DA, Anderson SR (1994) Dityrosine formation in calmodulin: conditions for intermolecular cross-linking. Biochemistry 33: 13363–13372

Malencik DA, Anderson SR (1996) Dityrosine formation in calmodulin: cross-linking and polymerization catalyzed by *Arthromyces* peroxidase. Biochemistry 35: 4375–4386

Malencik DA, Zhao ZZ, Anderson SR (1990) Determination of dityrosine, phosphotyrosine phosphothreonine, and phosphoserine by high-performance liquid chromatography. Anal Biochem 184: 353–359

Malencik DA, Sprouse JF, Swanson CA, Anderson SR (1996) Dityrosine: preparation, isolation, and analysis. Anal Biochem 242: 202–213

Marquez LA, Dunford HB (1995) Kinetics of oxidation of tyrosine and dityrosine by myeloperoxidase compounds I and II. Implications for lipoprotein peroxidation studies. J Biol Chem 270: 30434–30440

Meador WE, Means AR, Quiocho FA (1992) Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. Science 257: 1251–1255

Mukhopadhyay CD, Chatterjee IB (1994) NADPH-initiated cytochrome P450-mediated free metal ion-independent oxidative damage of microsomal proteins. Exclusive prevention by ascorbic acid. J Biol Chem 269: 13390–13397

O'Connell AM, Gieseg SP, Stanley KK (1994) Hypochlorite oxidation causes cross-linking of Lp(a). Biochim Biophys Acta 1225: 180–186

Olson NJ, Pearson RB, Needleman DS, Hurwitz MY, Kemp BE, Means AR (1990) Regulatory and structural motifs of chicken gizzard myosin light chain kinase. Proc Natl Acad Sci USA 87: 2284–2288

Pfeiffer S, Schmidt K, Mayer B (2000) Dityrosine formation outcompetes tyrosine nitration at low steady state concentrations of peroxynitrite. Implications for tyrosine modification by nitric oxide/superoxide *in vivo*. J Biol Chem 275: 6346–6352

Prütz WA, Butler J, Laud EL (1983) Phenol coupling initiated by one-electron oxidation of tyrosine units in peptides and histone. Int J Radiat Biol 44: 183–196

Santos CX, Bonini MG, Augusto O (2000) Role of the carbonate radical anion in tyrosine nitration and hydroxylation by peroxynitrite. Arch Biochem Biophys 377: 146–152

Savenkova ML, Mueller DM, Heinecke JW (1994) Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. J Biol Chem 269: 20394–20400

Smail EH, Briza P, Panagos A, Berenfeld L (1995) *Candida albicans* cell walls contain the fluorescent cross-linking amino acid dityrosine. Infect Immun 63: 4078–4083

Small EW, Anderson SR (1988) Fluorescence anisotropy decay demonstrates calcium-dependent shape changes in photo-cross-linked calmodulin. Biochemistry 27: 419–428

Spikes JD, Shen HR, Kopeckova P, Kopecek J (1999) Photodynamic crosslinking of proteins. III. Kinetics of the FMN- and rose bengal-sensitized photooxidation and intermolecular crosslinking of model tyrosine-containing N-(2-hydroxypropyl)methacrylamide copolymers. Photochem Photobiol 70: 130–137

Steiner RF, Albaugh S, Kilhoffer MC (1991) Distribution of separations in an engineered calmodulin. J Fluorescence 1: 15–22

Tew D, Ortiz de Montellano PR (1988) The myoglobin protein radical. Coupling of Tyr-103 to Tyr-151 in the H_2O_2-mediated cross-linking of sperm whale myoglobin. J Biol Chem 263: 17880–17886

Tilley KA, Benjamin RE, Bagorogoza KE, OkotKotber BM, Prakash O, Kwen H (2001) Tyrosine crosslinks: the molecular basis of gluten structure and function. J Agric Food Chem 49: 2627–2632

van der Vliet A, O'Neill CA, Halliwell B, Cross CE, Kaur H (1994) Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. FEBS Lett 339: 89–92

van der Vliet A, Eiserich JP, O'Neill CA, Halliwell B, Cross CE (1995) Tyrosine modification by reactive nitrogen species: a closer look. Arch Biochem Biophys 319: 341–349

Verweij H, Christianse K, Van Steveninck J (1982) Ozone-induced formation of O,O′-dityrosine cross-linked in proteins. Biochim Biophys Acta 701: 180–184

Weber G (1952) Polarization of fluorescence of macromolecules I: theory and experimental method. Biochem J 51: 145–155

Wells-Knecht MC, Huggins TG, Dyer DG, Thorpe SR, Baynes JW (1993) Oxidized amino acids in lens protein with age. Measurement of o-tyrosine and dityrosine in the aging human lens. J Biol Chem 268: 12348–12352

Wu W, Chen, Hazen SL (1999) Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. J Biol Chem 274: 25933–25944

Zhang H, Joseph J, Feix J, Hogg N, Kalyanaraman B (2001) Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: comparison with nitration and oxidation of tyrosine by peroxynitrite in aqueous solution. Biochemistry 40: 7675–7686

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