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DITYROSINE FORMATION IS IMPAIRED BY TYROSINE PHOSPHORYLATION

Seelos Christian[a,*], Gmeiner Bernhard[a], Rainer Patrizia[b] and Marian Brigitte[b]

[a]Institute of Medical Chemistry and [b]Institute of Tumorbiology
University of Vienna, A-1090 Vienna, Austria

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Summary: Using pure tyrosine and phosphotyrosine we have recently shown that phosphotyrosine is unable to form peroxidase catalyzed dimers (1989, FEBS Lett. 255, 395-397). In the present report, the effect of phosphotyrosine residues within a protein structure on dityrosine formation was studied using casein as a model protein. Dephosphorylation of casein resulted in a dose and time dependent increased synthesis of dityrosines following treatment with peroxidase/H_2O_2. The extent of crosslink formation was inversely related to the amount of phosphorylated tyrosine residues as quantitated by immunblotting. Thus, phosphorylation of tyrosine residues could play a regulatory role in protein-crosslinking where dityrosine bonds are involved.

Dityrosine (3,3-bityrosine) is the raction product of tyrosine oxidation [1,2]. Formation of dityrosine has been suggested to be important for inter- and intramolecular crosslinking of proteins [3-5]. Indeed, the occurrence of dityrosine has been reported in a variety of isolated proteins and in biological systems [6-11]. Most remarkably, this protein modification is beeing utilized in the sea-urchin egg. Rapidly following fertilization, increased oxygen consumption is used to generate H_2O_2 consumed by a secreted ovoperoxidase to link tyrosine residues and harden the protective fertilization envelope [reviewed in 12].

We have recently shown that the formation of dityrosine is impaired when tyrosine is covalently mofified by phosphorylation [13]. Since these results were obtained with pure tyrosine and

*To whom correspondence should be addressed at University of Vienna, Institute of Medical Chemistry, Währingerstrasse 10, 1090 Vienna, Austria.

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phosphotyrosine, we decided to investigate how the extent of tyrosine phosphorylation in a protein context influences its potential to form dityrosine links. We have chosen phosphocasein as a model protein because it is rich in tyrosine [5], can undergo crosslinking via dityrosine bonds [14] and has phosphotyrosine residues as we show by immunblotting using a highly specific anti-phosphotyrosine antibody. We demonstrate that dephosphorylation of phosphotyrosines in casein strongly enhances its ability to form dityrosines.

Materials and Methods

Materials: Casein was from E.Merck, Darmstadt, FRG. Horseradish peroxidase (HRP, EC 1.11.1.7, 200 U/mg) and calf intestine alkaline phosphatase (AP, EC 3.1.3.1, 140 U/mg) were purchased from Boehringer Mannheim, FRG. A mouse monoclonal anti-phosphotyrosine antibody (PY-69) was obtained from ICN Biochemicals, Cleveland, OH, USA. Peroxidase conjugated anti-mouse IgG was from DAKO, Denmark.

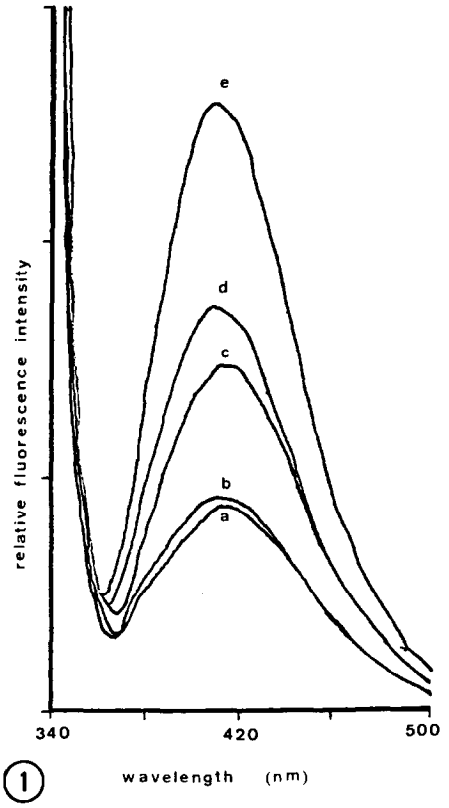
Dityrosine formation: The reaction mixture consisted of: 0.1 M sodium-borate, pH 8.0, 3.33 μM HRP, 1.5 mM H_2O_2 and 1 mg/ml of the respective casein preparation. Unless otherwise indicated, incubations were done for 15 min at 25°C. Dityrosine formation was monitored by fluorescence measurement according to [15] using a Hitachi 650-10S fluorescence spectrophotometer.

Dephosphorylation of casein: For enzymatic dephosphorylation, casein (1 mg/ml) was dissolved in 0.1 M borate buffer, pH 8.0, and treated with alkaline phosphatase at 30°C. At the appropriate time the phosphatase activity was quenched by 10 mM EDTA and dityrosine formation was estimated as described above.

Detection of phosphotyrosine: Phosphotyrosine residues in casein preparations were detected by a monoclonal anti-phosphotyrosine antibody. Samples (100 μl) of casein (1 mg/ml) were applied onto nitrocellulose using the slot blot apparatus (PR 600 Slot Blot) from Hoefer Scientific Instruments, San Francisco, CA, USA. After application the membranes were rinsed by suction with borate buffer according to the manufactures instructions. Membranes were blocked by TNE/BSA buffer (20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.05% TWEEN-20, 0.1 mM sodium-vanadate and 3% BSA) for 5 hours and incubated overnight at 4°C with anti-phosphotyrosine antibody (1:1000) in TNE buffer. After washing, the bound antibody was detected by incubation with peroxidase-linked anti-mouse IgG, AEC was used as a substrate for peroxidase. The relative amounts of bound anti-phosphotyrosine antibody was estimated by scanning the immunblots with a Beckman CDS-200 densitometer at 570 nm.

Results

Protein dityrosine synthesis in vitro can be catalyzed by peroxidases, uv-irradiation, gamma-irradiation or oxygen radicals [16]. When phosphocasein was incubated in the presence



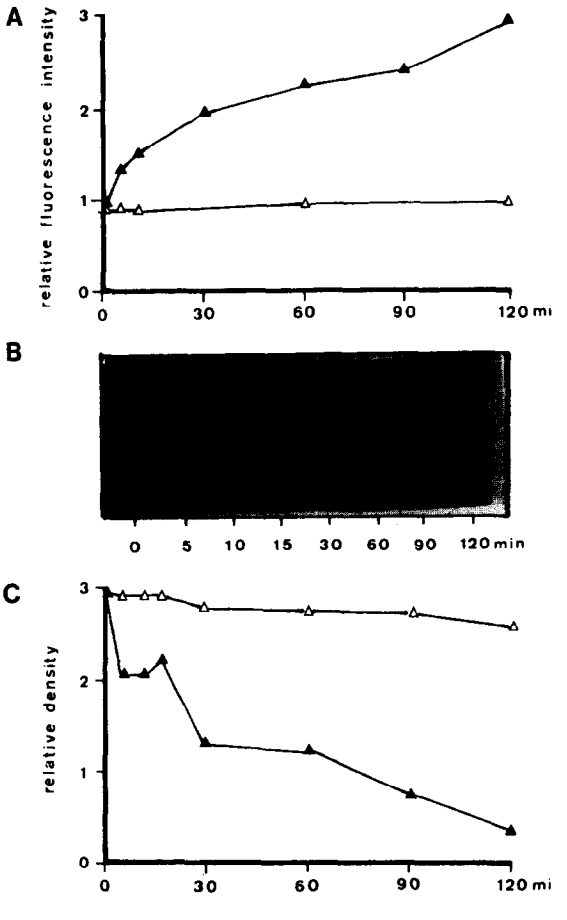


Figure 1. Dityrosine-fluorescence emission spectra of casein. Casein was treated as given in the materials and methods section to generate dityrosine-crosslinks. Untreated casein (a); casein preincubated with phosphatase: 1 U/ml (b), 2 U/ml (c); 5 U/ml (d) and 10 U/ml (e), at 30°C for 30 minutes.

Figure 2. Dityrosine formation and phosphotyrosine immunreactivity of casein during dephosphorylation. Casein was incubated in the absence or presence of 2 U/ml phosphatase at 30°C up to 120 minutes. At the indicated times samples were analyzed for dityrosine formation (as calculated from the fluorescence emission intensities) and phosphotyrosine immunreactivity as described in materials and methods. A: dityrosine formation (Δ) no phosphatase, (▲) 2 U/ml phosphatase. B: phosphotyrosine immunblot; upper row: no phosphatase; lower row: 2 U/ml phosphatase. C: densitometric scan of phosphotyrosine immunblot (Δ) no phosphatase, (▲) 2 U/ml phosphatase.

of HRP and H_2O_2 for 15 min at 25°C, dityrosine was formed (Fig. 1). The fluorescence spectrum of the reaction mixture was identical to spectra reported for pure dityrosine or dityrosine present in various proteins exposed to HRP/H_2O_2 [5,15]. To determine if the casein we used contained phosphorylated tyrosine residues, immundetection by an anti-phosphotyrosine

antibody was performed. As shown in Fig. 2B, the antibody clearly detected the presence of phosphotyrosines in the casein solution. Thus, dephosphorylation of casein should increase the number of tyrosyl residues susceptible for dityrosine formation. Fig. 1 illustrates how treatment of casein with increasing activities of phosphatase (1-10 U/ml) for 30 min at 30°C followed by HRP/H_2O_2 incubation resulted in a striking dose dependent increase in dityrosine production. At 5 U phosphatase/ml a two-fold and at 10 U/ml a 3.5-fold increase was observed. To demonstrate that this treatment indeed decreased the number of phosphorylated tyrosine residues, we incubated casein with 2 U of phosphatase/ml at 30°C up to 120 min. At the indicated times (Fig. 2) samples were withdrawn to estimate dityrosine formation and phosphotyrosine content. Fig. 2 clearly shows that, as dityrosine formation increased by phosphatase treatment, the amount of immunreactive phosphotyrosine declined. In control incubations without phosphatase, the extent of dityrosine formation and the amount of immunreactive material was virtually unchanged (Fig. 2).

Discussion

Applying peroxidase catalyzed in-vitro synthesis of dityrosines we were able to study regulatory aspects of this mechanism. We found that a strong correlation existed between the extent of phosphorylation of tyrosine residues and a protein's potential to form dityrosines. Dephosphorylation of casein with alkaline phosphatase removed phosphate from tyrosine residues as shown by immundetection with an anti-phosphotyrosine antibody. This was paralleled by an enhanced ability to form dityrosines after treatment with peroxidase/H_2O_2. Although calf intestine alkaline phosphatase shows relative specifity for phosphorylated tyrosine residues [17], some phosphoserine or -threonine residues might be dephosphorylated as well. However, this should not have any direct influence on the synthesis of dityrosines. Clearly, our present findings corroborate our recent results made with pure tyrosine or phosphotyrosine solutions and extend these to a protein environment.

Tyrosine phosphorylation of proteins has gained much attention by researchers dealing with the control and regulation of cell growth in normal and malignant cells. Yet, it is often unclear how tyrosine phosphorylation affects the activities and

functions of these proteins [18]. Considering our recent [13] and present observations it is tempting to speculate that phosphorylation/dephosphorylation of tyrosine residues in a particular protein might regulate its ability to form dityrosine-crosslinks.

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