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Phosphorylation of tyrosine prevents dityrosine formation in vitro

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Treatment of L-tyrosine in a peroxidase/H_2O_2 system results in the formation of dityrosine. However, the phosphoester derivative of tyrosine, *O*-phospho-L-tyrosine, was unable to form dityrosine even in mixtures with free L-tyrosine. Dephosphorylation of *O*-phospho-L-tyrosine by alkaline phosphatase followed by horseradish peroxidase/H_2O_2 treatment resulted in the formation of dityrosine. Our in vitro results indicate that phosphorylation/dephosphorylation of L-tyrosine may regulate dityrosine formation, and is supposed to play an important role in protein–protein interactions, i.e. crosslinking.

Tyrosine; Dityrosine; Phosphotyrosine

1. INTRODUCTION

Dityrosine (3,3′-bityrosine) is the reaction product of tyrosine oxidation which can be catalyzed by peroxidases [1,2]. During the process of tyrosine dimerization, tyrosine radicals are formed [3]. The presence of naturally occurring dityrosines has been reported for structural and globular proteins and dityrosine formation has been suggested to be responsible for inter/intramolecular crosslinks of polypeptides and proteins [4–6]. Further biological significance of dityrosine occurrence has been described for the process of hardening of the sea urchin egg membrane after fertilization, an ovoperoxidase-driven process [7,8]. Recently, Briza et al. [9], reported on the identification of dityrosine as a prominent component of the yeast ascospore wall, where it is suggested to be an important factor in improving the mechanical stability of the spores.

Tyrosine can be covalently modified by phosphorylation and it has been demonstrated and suggested that protein-tyrosine phosphorylation/dephosphorylation may play a key role in cell growth and differentiation [10,11].

Referring to these reports, we therefore tested tyrosine and phosphotyrosine on their ability to form dityrosine, taking advantage of an established in vitro HRP/H_2O_2-catalyzed dityrosine forming system [2,3].

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2. MATERIALS AND METHODS

2.1. *Materials*

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. L-tyrosine was from E. Merck, Darmstadt, FRG and *O*-phospho-L-tyrosine from Sigma Co., St. Louis, MO, USA.

2.2. *Dityrosine formation*

The reaction mixture consisted of: PBS, pH 7.4, 3.33 μM HRP, 1.5 mM H_2O_2 and the respective concentrations of L-tyrosine and/or *O*-phospho-L-tyrosine. Incubations were done for 10 min at 25°C. Dityrosine formation was monitored and calculated according to [2].

2.3. *Dephosphorylation of O-phospho-L-tyrosine*

O-phospho-L-tyrosine was dissolved in 50 mM Tris-HCl, pH 8.8, and treated with alkaline phosphatase (0.5–4.0 U/ml) at 37°C. At the end of incubation, the phosphatase activity was quenched by 10 mM EDTA and dityrosine formation was estimated as given above. Release of inorganic phosphate was measured according to [12].

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3. RESULTS AND DISCUSSION

When tyrosine was incubated in the presence of HRP and H_2O_2 for 10 min at 25°C, dityrosine was formed (fig.1). The peroxidase catalyzed dimerization of tyrosine was first described by Gross and Sizer [1] and it has recently been shown that the reaction is mediated via a tyrosine-cation radical [3]. In contrast to tyrosine, virtually no dityrosine was present after 10 min at 25°C, when phosphotyrosine was treated with HRP and H_2O_2 (fig.1). Since this result might be due to a direct inhibitory effect of phosphotyrosine on HRP activity, mixing experiments were performed. As seen in table 1, in tyrosine/phosphotyrosine mixtures (molar ratios 1:1 and 1:2), dityrosine was still formed to an amount also found in parallel incubations containing only unphosphorylated tyrosine.

The results show that phosphotyrosine does not inactivate the HRP/H_2O_2 system and is also unable to form dityrosine by dimerization with tyrosine. Therefore, we conclude that blocking of the phenolic OH-group of tyrosine by the phosphate moiety abolishes the potential of tyrosine to undergo peroxidase-catalyzed dimerization.

Considering the concerted action of tyrosine kinases and phosphatases in regulation of protein phosphorylation reactions, the dephosphorylation of phosphotyrosine should be able to restore the molecule's ability to form dityrosine. For this purpose, calf intestine alkaline phosphatase was chosen because this phosphatase showed selectivity for phosphorylated tyrosine residues [13]. As illustrated in fig.2, the treatment of phosphotyrosine (2.5 mM) by calf intestine alkaline phosphatase (0.5–4 U/ml) for 10 or 20 min at 37°C, followed by HRP/H_2O_2 incubation resulted in a time- and concentration-dependent increase in dityrosine formation which was paralleled by a time- and concentration-dependent release of P_i (fig.2 insert) indicating the

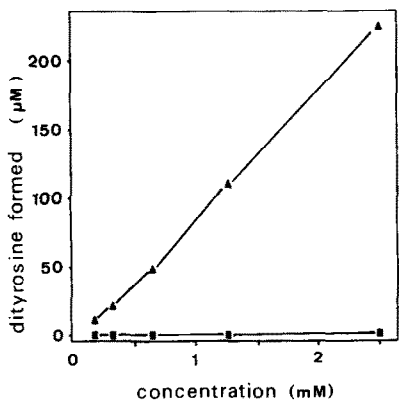

Fig.1. Dityrosine formed from L-tyrosine (▲) or *O*-phospho-L-tyrosine (■) at the indicated concentrations after 10 min at 25°C. Formation of dityrosine was estimated as described in section 2.

Table 1

Dityrosine formation in mixtures of L-tyrosine and *O*-phospho-L-tyrosine

Compound	Concentration (mM)	Dityrosine formed (μM)
Tyrosine	0.625	53
Tyrosine	1.25	143
Tyrosine	1.875	214
Phosphotyrosine	0.625	0
Phosphotyrosine	1.25	0
Tyrosine/phosphotyrosine	0.625/0.625	60
Tyrosine/phosphotyrosine	0.625/1.25	52

Tyrosine, phosphotyrosine and mixtures of tyrosine and phosphotyrosine were treated with HRP/H_2O_2 and dityrosine formation was estimated as given in section 2

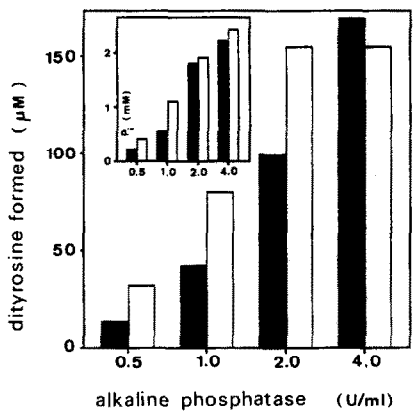

Fig.2. Dityrosine formed from *O*-phospho-L-tyrosine during dephosphorylation. Phosphotyrosine (2.5 mM) was treated with indicated activities of alkaline phosphatase for either 10 (black bars) or 20 (white bars) min at 37°C, followed by addition of HRP/H_2O_2 as described in section 2. The insert shows the liberation of P_i in the incubation mixtures consisting of *O*-phospho-L-tyrosine (2.5 mM) and indicated activities of alkaline phosphatase after 10 (black bars) or 20 (white bars) min at 37°C. P_i concentrations were measured as given in section 2.

liberation of 'HRP/H_2O_2-reactive' tyrosine by the phosphatase.

Our present results clearly show that in contrast to tyrosine, phosphotyrosine does not undergo dimerization but retains its ability to form dityrosine after dephosphorylation by intestine alkaline phosphatase. Since dityrosine formation is a naturally occurring process influencing the physicochemical properties of proteins, the existence of a regulatory system may be important for the maintenance of normal function. If the action of protein-tyrosine kinases and phosphotyrosyl-protein phosphatases is at least partly involved in the regulation of protein crosslinking via dityrosine formation remains to be cleared.

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