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Dimers formation by cytochrome c-catalyzed oxidation of tyrosine and enkephalins

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Summary. Cytochrome c in the presence of H_2O_2 is able to oxidize tyrosine and enkephalins into the corresponding dimers linked by a o,o′-biphenyl link, as evidenced by the spectral and HPLC analysis. Also amino acid analysis of the hydrolysates from cytochrome c-treated enkephalins provides evidence for the presence of dityrosine. Kinetics of the oxidation was followed both spectrophotometrically and fluorometrically. The reaction rate depends on cytochrome c and hydrogen peroxide concentrations. For the first time it is demonstrated that the o,o′-biphenyl link of dityrosine can be formed by the action of an enzymatic system alternative to peroxidase. Some general considerations on the possible consequences of these reactions inside the mitochondrion are outlined.

Keywords: Amino acids – Dityrosine – Enkephalin – Cytochrome c – Hydrogen peroxide

Introduction

Dityrosine is an unusual aminoacid formed by the o,o′-biphenyl link between two residues of tyrosine. It can be produced by the action of peroxidase in the presence of hydrogen peroxide (Bayse et al., 1972) or by the effect of the hydroxyl radical (Lubec et al., 1994), ultraviolet irradiation (Malencick and Anderson, 1987), NO_2 (Kikukawa et al., 1994) and peroxynitrite (van der Vliet et al., 1995). The aminoacid has been discovered in several proteins (Andersen, 1964; Bailey, 1991) and it contributes together with other compounds, such as carbolines, to the cross-link of proteins (Wells-Knecht et al., 1993). Oxidative coupling of tyrosine occurs also in several invertebrate systems forming insoluble structural proteins such as the cuticular one (Fetterer et al., 1990). Dityrosine can be also considered as an age-dependent marker of the oxidative damage inside the cell (Bailey, 1991). Since the o,o′-biphenyl

link is very stable, its presence could represent probably an irreversible factor of modification in the cell.

Enkephalins are pentapeptides with opiate-like activity (Hughes et al., 1975); their role as neurotransmitters has been widely investigated (Smith et al., 1976) and it has been reported that their action is rapidly removed by peptidases (Hambrook et al., 1976). Recently we have started a series of investigations on alternative metabolic pathways for enkephalins. Actually we have found that enkephalins are substrates for tyrosinase (Rosei et al., 1989) giving rise to soluble melanopeptides collectively named opiomelanins (Rosei et al., 1992; 1994; 1995). Another alternative route of enkephalin oxidation is represented by the oxidative ring coupling by means of amino terminal tyrosine catalyzed by the peroxidase/H_2O_2 system which leads to the formation of enkephalin dimers linked by a dityrosine residue (Rosei et al., 1991a). It may be recalled that enkephalin dimers have a precise physiological activity in the cell, being able to suppress the respiratory burst of polymorphonuclear leucocytes (Rabgaoui et al., 1993).

Since it has been recently reported that cytochrome c in the presence of hydrogen peroxide can catalyze the oxidation of some organic molecules (Radi et al., 1991) we have undertaken experiments on the possible tyrosine ring coupling by means of cytochrome c/H_2O_2 system. In this paper we present evidence that tyrosine and enkephalins can function as hydrogen donors for cytochrome c/H_2O_2 system leading to the formation of dityrosine or enkephalin dimers.

Materials and methods

Materials

Leu-enkephalin (Leu-enk), Met-enkephalin, Kyotorphin, Tyr-Gly, Tyr-Gly-Gly and cytochrome c from horse heart were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). All other reagents were analytical grade products from Merck (D-6100 Darmstadt, Germany). Dityrosine was prepared according to the procedure of Amadò et al. (1984). Hydrogen peroxide dilute solutions were freshly prepared every day; the concentration was tested using the molar extinction coefficient of 72.4 at 230 nm (Nelson and Kiesow, 1972).

Kinetic measurements

Kinetic measurements were performed by recording the rate of dityrosine formation at 315 nm (Bayse et al., 1972). The absorbance was followed continously using a Varian DMS 200 spectrophotometer in 1 cm light path thermostated cuvettes at 25°C. Dityrosine formation was also followed by the increase in fluorescence using excitation wavelenght of 285 nm and emission wavelenght of 405 nm, with 5 nm band width, in a Jasco Spectrofluorometer FP770, using 1 cm thermostated cuvettes at 25°C. Unless otherwise stated, the reaction mixture contained: 0.5 mM substrate, 4 mM H_2O_2 and 3.2 μM cytochrome c in 0.1 M Tris-HCl buffer, pH 8.0. The reaction was started by H_2O_2 addition. Parallel samples without cytochrome c or with heat-inactivated protein were used as controls. In absence of H_2O_2 cytochrome c did not carry out the reaction. The steady-state rate was defined as the slope of the linear zone of the product accumulation curve.

Chromatographic analysis

For chromatographic analysis, the incubation mixture contained 2 mM Leu-enk, 10 mM hydrogen peroxide and 64 μM cytochrome c; the experiments were carried out at 37°C in 0.2 M sodium borate buffer, pH 9.5, according to Amadò et al. (1984).

For dityrosine determination, aliquots of incubation mixture were drawn at various times and the reaction was stopped by the addition of trichloroacetic acid to 10% final concentration. After centrifugation, the samples were hydrolyzed in 6N HCl in sealed tubes at 110°C for 20 h. Hydrolysates were analyzed in a Carlo Erba Amino acid analyzer mod. 3A30, using a 12 × 0.46 cm column, filled with 3AR/IC/6/10 resin. The column was equilibrated with 0.15 M sodium citrate buffer, pH 3.3, and eluted with A) 0.15 M sodium citrate buffer, pH 3.3, for 10 min, B) 0.2 M sodium citrate buffer, pH 4.13, for 14 min and C) 1.2 M sodium citrate buffer, pH 5.9, for 15 min, at 48°C for 8 min, then at 70°C. The flow rates of buffer and ninhydrin were 30 and 20 ml/h, respectively. In the employed conditions, dityrosine was eluted as a well separated peak with retention time of 35 min.

For dimers analysis, the same incubation mixture was employed. After incubation at 37°C an aliquot of the mixture was treated with sodium metabisulfite to destroy hydrogen peroxide, filtered and injected on a reverse-phase column Novapak C18, 4μ, 15 × 0.39 cm, of a HPLC Waters-Millipore apparatus (Milford, MA, USA). The chromatography was carried out in isocratic conditions at 25°C in thermostated apparatus, utilizing as eluent a mixture containing 85% 0.05 M sodium citrate-phosphate buffer, pH 5.0, and 15% acetonitrile, with a flow rate of 1.5 ml/min.

Results

When Leu-enk is oxidized by the cytochrome c/H_2O_2 system, an absorption spectrum with maxima at 290 and 315 nm quickly develops; this spectral pattern can be attributed to a biphenyl bond between two tyrosine residues (Amadò et al., 1984; Andersen, 1964; Verweij et al., 1982).

Kinetic measurements indicate that the oxidation rate of Leu-enk is linear towards cytochrome c concentration (Fig. 1); when cyanide is added to the reaction mixture a drastic inhibition occurs, indicating that cytochrome c is responsible for the reaction. The reaction follows a Michaelis-Menten kinetics for all the substrates tested; in Table 1 the kinetic parameters for the oxidation of various peptides having an amino terminal tyrosine residue are reported, compared to those of tyrosine. The best substrates are Leu- and Met-enkephalin; however also the tripeptide Tyr-Gly-Gly and the two dipeptides Tyr-Gly and kyotorphin (Tyr-Arg) are better substrates than tyrosine for the cytochrome c/H_2O_2 system. This result is in good agreement with previous experiments indicating the favourable effect of the peptidic bond for the oxidation of phenolic ring (Rosei et al., 1991a; Foppoli et al., 1991) and confirms that in the presence of hydrogen peroxide the catalysis of oxidative and peroxidative enzymes acting on peptides is improved as the chain lenght is longer (Rosei et al., 1989; Rosei et al., 1991b). Identical results were obtained following the dityrosine production by the increase in fluorescence emission at 405 nm upon excitation at 285 nm.

The reaction follows the Michaelis Menten kinetics also for hydrogen peroxide (Fig. 2). The Lineweaver-Burk plot used to investigate the effect of

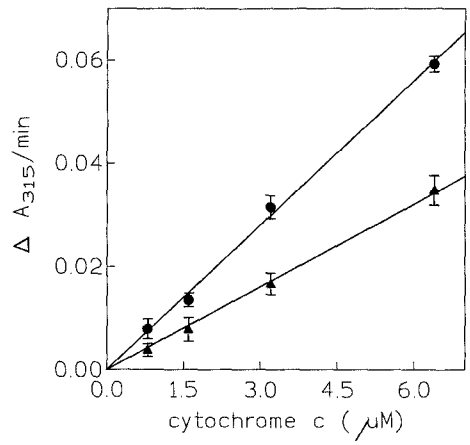

Fig. 1. Leu-enk oxidation by cytochrome c/H_2O_2 as a function of cytochrome c concentration, in absence (circles) or presence (triangles) of CN^-. Incubation mixture contained 0.5 mM Leu-enk, 4 mM hydrogen peroxide and cytochrome c at the indicated concentrations in 0.2 M Tris-HCl buffer, pH 8.0. Cyanide, when present, was 0.15 mM. The reaction rate is expressed as dimer formation, measured by the increase of absorbance/min at 315 nm

Table 1. Kinetic parameters for opioid peptides oxidation by cytochrome c/H_2O_2 system

Substrate	Km (mM)	Vmax (ΔA/min at 315 nm)	Kcat (Vmax/mg cyt.c)	Kcat/Km
Leu-enkephaklin	0.349 ± 0.052	0.060 ± 0.003	1.50 ± 0.075	4.3
Met-enkephalin	0.356 ± 0.010	0.085 ± 0.009	2.12 ± 0.220	6.0
Tyr-Gly-Gly	0.489 ± 0.024	0.079 ± 0.017	1.97 ± 0.420	4.03
Kyotorphin	0.683 ± 0.091	0.110 ± 0.007	2.75 ± 0.175	4.03
Tyr-Gly	0.920 ± 0.090	0.174 ± 0.021	4.35 ± 0.520	4.73
Tyrosine	1.189 ± 0.140	0.157 ± 0.013	3.92 ± 0.320	3.3

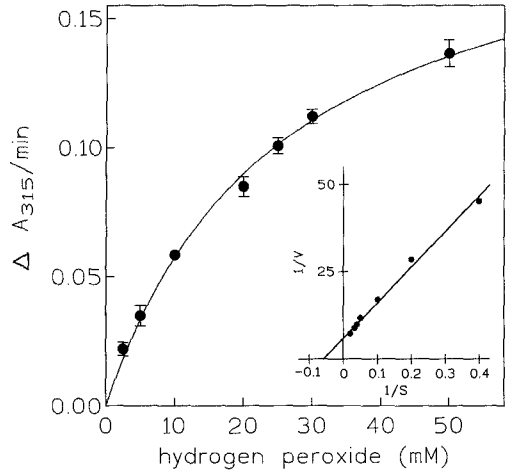

Fig. 2. Reaction rate of Leu-enk oxidation by cytochrome c/H_2O_2 as a function of H_2O_2 concentration. Inset: Lineweaver-Burk plot of the same reaction. Incubation mixture contained 0.5 mM Leu-enk, 3.2 μM cytochrome c and hydrogen peroxide at the indicated concentrations in 0.2 M Tris-HCl buffer, pH 8.0. The rate is expressed as dimer formation, measured by the increase of absorbance/min at 315 nm

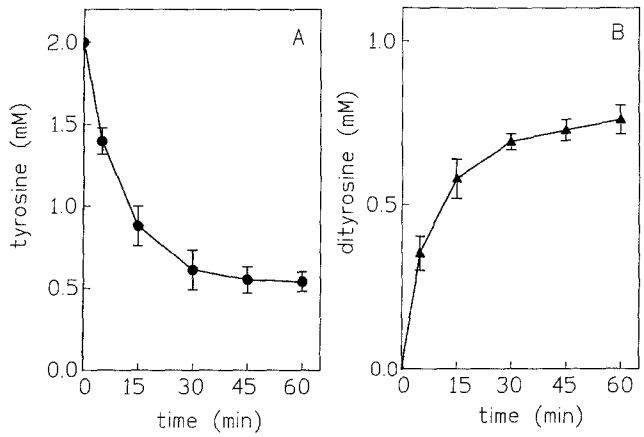

Fig. 3. Tyrosine decay (**A**) and dityrosine formation (**B**) from Leu-enk oxidation by cytochrome c/H_2O_2 as a function of incubation time. Incubation mixture contained 2 mM Leu-enk, 10 mM hydrogen peroxide and 64 μM cytochrome c in 0.2 M sodium borate buffer, pH 9.5. The mixture was incubated at 37°C; the reaction was stopped at various times by the addition of trichloroacetic acid to precipitate cytochrome c. After centrifugation, the samples were hydrolyzed and subjected to amino acid analysis. For details see Materials and methods

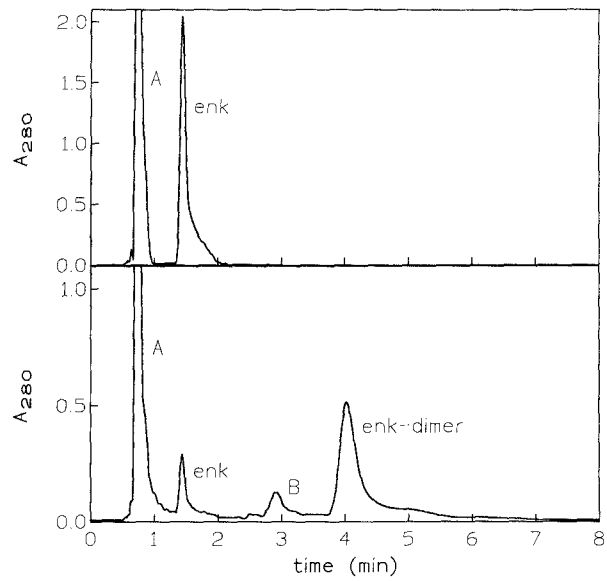

Fig. 4. HPLC profile of Leu-enk incubated for 0 (upper) or 20 min (lower) with cytochrome c/H_2O_2. Incubation mixture contained 2 mM Leu-enk, 10 mM hydrogen peroxide and 64 μM cytochrome c in 0.2 M sodium borate buffer, pH 9.5. After incubation at 37°C the reaction was stopped by addition of sodium metabisulfite and the mixture was filtered and analyzed. For details see Materials and methods. Peak A is due to sodium metabisulfite, while peak B could be a secondary product from enkephalin peroxidation

variation of hydrogen peroxide on the rate of dimer production (inset Fig. 2) displays an apparent Km value of 25 mM, that is in agreement with that reported by Radi et al. (1991).

In order to demonstrate the synthesis of the enkephalin dimers linked by a dityrosine residue, a hydrolysis of Leu-enk incubated for various times with the cytochrome c/H_2O_2 system was carried out and the aminoacidic content of the hydrolysates was determined. While the content of Gly, Phe and Leu is unchanged at any time, a diminution of Tyr as a function of incubation time is observed (Fig. 3A). At the same time the concomitant formation of dityrosine can be evaluated (Fig. 3B). About 70% of tyrosine present in the enkephalin molecule is converted into dityrosine.

Fig. 4 shows the HPLC profiles at zero time (upper) and after 20 min incubation (lower) of mixtures containing Leu-enk as substrate. The peak with retention time of 1.5 min represents Leu-enk, as determined by comparison with a standard sample. In the lower chromatogram it is evident the drastic fall of this peak and the concomitant appearance of another peak with retention time of about 4 min. The eluate corresponding to this peak was collected, hydrolyzed and analyzed for aminoacid composition; the presence of dityrosine and its relative amount with respect to the other amino acids forming the enkephalin lead to the conclusion that the peak represents the dimer of Leu-enk.

Discussion

Our results demonstrate that cytochrome c/H_2O_2 system is able to oxidize tyrosine and enkephalins into the corresponding dimers linked by an o,o′-biphenyl bond. Reaction assays under the experimental conditions used showed that the product formation was linear for at least ten minutes. Among the substances tested, enkephalins are the best substrates and this result confirms that the presence of peptidic bonds is, as already demonstrated (Rosei et al., 1991a), a favourable factor for the oxidation of the phenolic ring. This result can be explained considering that the peptidic bond can facilitate the formation of the radical on the tyrosine residue, whose presence is necessary for the coupling reaction.

The dityrosine o,o′-biphenyl link, which is very stable, occurs in many proteins throughout the animal kingdom and can contribute to stabilize the protein structure; on the other hand it can also be considered as a parameter of aging (Bailey, 1991). As far as we know the enzymatic oxidation of tyrosine to form the o,o′-biphenyl link has been shown to be carried out only by peroxidases. Our results demonstrate for the first time that the reaction can be carried out also by cytochrome c.

It is well known that mitochondria are able to produce a considerable amount of H_2O_2 (Boveris et al., 1972) and the ability of cytochrome c to catalyze the dityrosine formation suggests that such dimerization could be ultimately performed also inside the mitochondria in the presence of suitable hydrogen donors.

As this type of dimerization occurs among proteins and peptides, the presence of hydrogen peroxide inside the mitochondria and expecially its amount could be a favourable factor to create cross-links inside the organelle and ultimately represent a cause of toxicity and aging.

In our opinion these phenomena could be expecially important in all the pathophysiological conditions in which the level of H_2O_2 is enhanced. This event is a direct consequence of the catalase decrease that actually occurs in the cell during aging (Ben-Schachar and Youdim, 1990). Though the cytochrome c affinity for H_2O_2 is not very high, it should be taken into account that hydrogen peroxide is a common product generated inside the mitochondrion and its amount can remarkably increase as a consequence of pathological conditions. Our results seem to indicate some interesting features as at present the mitochondrial space is considered a primary site for the induction of brain aging (Fhan and Cohen, 1992).

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