Journal of Photochemistry and Photobiology A: Chemistry 198 (2008) 111–118

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Competitive photosensitized oxidation of tyrosine and methionine residues in enkephalins and their model peptides

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ARTICLE INFO

Article history:
Received 17 January 2008
Received in revised form 21 February 2008
Accepted 28 February 2008
Available online 7 March 2008

Keywords:
Enkephalins
Sensitized photooxidation
Quenching
Electron transfer
Laser flash photolysis

ABSTRACT

Laser flash photolysis was used to investigate the oxidation of methionine (Met) and tyrosine (Tyr) residues in methionine-enkephalin (MetEnk, TyrGlyGlyPheMet) by the triplet state of 4-carboxybenzophenone (4CB). Quenching of the 4CB triplet by model amino acids and peptides (Tyr, TyrGly, TyrGlyGly, PheMet, TyrMet, MetTyr, MetEnk and TyrGlyGlyPheLeu) occurred with rate constants close to the diffusion-controlled limit, $k_q = (1-3) \times 10^9\ M^{-1}\ s^{-1}$. Experimental transient spectra, that were resolved into components, revealed the presence of various electron-transfer intermediates, i.e. ketyl radicals ($CBH^\bullet$) and ketyl radical anions ($CB^{\bullet-}$) of 4CB, tyrosyl radicals ($TyrO^\bullet$), and $(S_\therefore N)^+$ radical cations. Based on the concentration profiles obtained from spectral deconvolutions, quantum yields of the transients were determined. For the quenchers containing only the tyrosine residue, the quantum yields of the tyrosyl radicals were found to be close to unity and equal to the sum of the quantum yields for the formation of 4CB ketyl radicals and 4CB ketyl radical anions. For quenchers containing both tyrosine and methionine residues, the formation quantum yields of tyrosyl radicals were decreased from the value of 1 to approximately 0.7–0.8 which was equal to the sum of the quantum yields for the formation of $CBH^\bullet$ and $CB^{\bullet-}$. The above observations are discussed in terms of competitive oxidation reactions involving Tyr and Met followed by the intramolecular electron transfer from the tyrosine residue to the sulfur-centered radical cation on the methionine residue.

1. Introduction

Enkephalins, i.e. methionine-enkephalin (MetEnk, TyrGlyGlyPheMet) and leucine enkephalin (LeuEnk, TyrGlyGlyPheLeu), are endogenous pentapeptides exhibiting opiate-like activity. They have an influence on the functioning of the central nervous and immune systems [1]. Due to the presence of methionine and tyrosine residues, these peptides are prone to oxidation. Similar to the methionine in the β-amyloid peptide [2], the methionine residue in the enkephalin molecule undergoes metal-catalyzed oxidation, as demonstrated by EPR measurements [3]. The enzymatic and radical-mediated oxidation reactions of enkephalins, as well as their reduction by hydrogen atoms, have also been thoroughly studied [4–8].

In pulse radiolysis studies on proteins and peptides containing both methionine and tyrosine residues, Prütz et al. [9,10] have shown that there was a selective initial oxidation of methionine by $Br_2^{\bullet-}$ but that this oxidation was followed by a transformation of the methionyl radicals into tyrosyl radicals. For Met-enkephalin such a process was reported with a rate constant equal to ca. $5 \times 10^5\ s^{-1}$ [10,11], for Tyr-Met the rate constant was determined to be $1 \times 10^5\ s^{-1}$ [12], whereas for Met-Tyr the rate constant was $4 \times 10^4\ s^{-1}$ [9]. In the proline-bridged peptides, the rate constants of such intramolecular electron-transfer reactions decreased with the number of proline residues. Electron transfer occurred both through the peptide backbone and through direct and/or water mediated contact between the groups bearing the radical sites [12]. In conformationally flexible enkephalins, a correlation between the rate constants for electron transfer and the number of amino acids separating the methionine and tyrosine residues was not possible. For radical-initiated oxidation of Met-enkephalin, an electron-tunneling mechanism for intramolecular electron transfer from the Tyr to the radical site on the Met residue was postulated [11].

Although radical-initiated oxidation of enkephalins has been thoroughly studied, no information is available on photosensitized oxidation processes. However, it is well demonstrated that both tyrosine and methionine, investigated as single amino acids, exhibit very similar reactivity towards triplet states of carbonyl compounds, i.e. benzophenone (BP) and 4-carboxybenzophenone (4CB). In these reactions, the initial step for the Tyr or Met oxidations is an electron-transfer process [13–15]. As studied for the methionine residues in tyrosine-free peptides, a radical-ion

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doi:10.1016/j.jphotochem.2008.02.027

pair [4CB$^{\bullet-}\cdots$Q$^{\bullet+}$] is formed between the photosensitizer and the quencher (Q) which decays competitively by a back electron-transfer process, by the separation of the radical ions, and by a proton transfer within the radical-ion pair with a subsequent separation of these neutral radicals. The ratio between these three decay channels varies depending on the quencher [16,17]. Electron transfer from tyrosine to the triplet state of benzophenone yields radical ions, i.e. the tyrosine radical cation and the benzophenone radical anion [13]. Due to the low pK_a of the tyrosine radical cation (p$K_a = -1$) [18,19], there is an immediate deprotonation to the tyrosyl radical in aqueous solutions [20].

In the present paper we report on our research concerning the photosensitized oxidation of methionine-enkephalin and its model peptides in aqueous solution in the presence of the 4-carboxybenzophenone (4CB) as a sensitizer. Laser flash photolysis was used to generate the 4-carboxybenzophenone triplet state and to monitor its time behavior in the presence of various peptide quenchers as well as to detect other transients. With this technique, triplet-quenching rate constants and quantum yields for the transients were determined. These data are discussed in terms of competitive oxidation reactions involving Tyr and Met followed by the intramolecular electron transfer from the tyrosine residue to the sulfur-centered radical cation on the methionine residue.

2. Materials and methods

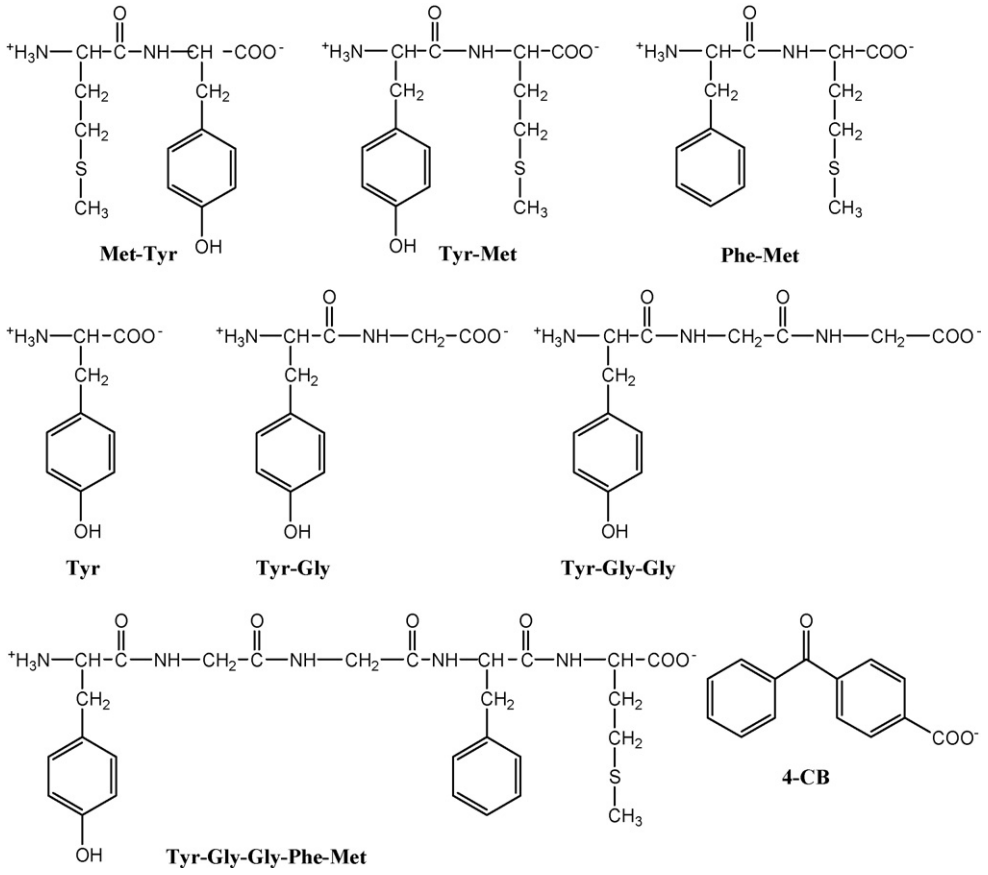
2.1. Chemicals

Leucine enkephalin (LeuEnk), methionine-enkephalin acetate salt (MetEnk), tyrosine (Tyr), and the dipeptides: tyrosyl-glycine (Tyr-Gly) and methionyl-tyrosine (Met-Tyr) (purity 99%) were all purchased from Bachem. Phenylalanine (Phe) and 4-carboxybenzophenone of 99% purity were obtained form Aldrich. The dipeptide phenylalanyl-methionine (Phe-Met) and the tripeptide tyrosyl-glycyl-glycine (Tyr-Gly-Gly) (purity 99%) came from Sigma. The dipeptide L-tyrosyl-L-methionine (Tyr-Met) of highest purity was synthesized by Research Plus Inc., Bayonne and Denville, NJ in USA. The structures of the compounds used in the experiments are presented in Scheme 1. Isopropanol (*i*PrOH) with 99.9% purity was purchased from Merck. The distilled water used in all the experiments was treated with a Millipore Milli-Q Plus system.

2.2. Laser flash photolysis

Nanosecond laser flash photolysis experiments were carried out with two different experimental setups: (i) at the facility of the Interdisciplinary Group of Time-Resolved Spectroscopy (University of Leipzig, Leipzig, Germany) and (ii) at the facility of the Ultrafast Laser Spectroscopy Center (Adam Mickiewicz University, Poznan, Poland).

(i) The nanosecond laser flash photolysis setup was described in detail elsewhere [21]. Laser excitation at 266 nm and 355 nm from a Quanta-Ray GCR-11 Nd:YAG laser (Spectra Physics) (operated at 3 mJ, pulse width 3 ns) was at a right angle with respect to the monitoring light beam. The optical detection system consisted of a pulsed Xenon lamp (XBO 150, Osram), a monochromator (SpectraPro-275, Acton Research), a R955 photomultiplier tube (Hamamatsu Photonics) or a fast Si-photodiode with 1 GHz amplification, and a 500 MHz digitizing oscilloscope (DSA 602 A, Tektronix). The power of every pulse of the laser was determined by



Scheme 1.

monitoring a scattered fraction of the beam with a fast Si photodiode. During irradiation, samples were flowed continuously through a quartz cell with an optical path length of 0.5 cm. All solutions were deoxygenated by bubbling high-purity N_2 for 15 min prior to the experiment and also during the measurements.

(ii) Laser excitation at 355 nm from a Q-switched Nd:YAG laser (Continuum Surelite II), operated at 3 mJ with a repetition rate of 0.5 Hz and a pulse width of 8 ns, was at a right angle with respect to the monitoring light beam. A 150 W xenon arc lamp (Applied Photophysics) operated at 1 Hz repetition rate was used as the source of the analyzing light. After leaving the cell, the monitoring light was dispersed by a monochromator and detected with a photomultiplier (R928 Hamamatsu) coupled to a digital oscilloscope (Tektronix TDS 680C). A detailed description of the nanosecond laser flash photolysis setup can be found elsewhere [22]. Experiments were performed in rectangular quartz cells (1 cm × 1 cm). All the samples were deoxygenated by bubbling high-purity argon for 20 min prior to the measurement.

All experiments were performed with freshly prepared alkaline or neutral solutions at room temperature. The pH was adjusted with hydrochloric acid and sodium hydroxide and measured with a 540GLP pH-meter (WTW). 4-Carboxybenzophenone was used at concentrations of ca. 4×10^{-3} M (λ_{ex} = 355 nm) and ca. 2×10^{-4} M (λ_{ex} = 266 nm). Concentrations of the quenchers ranged from 5×10^{-5} M to 5×10^{-4} M (for enkephalins only up to 4×10^{-5} M due to their low solubility). In the presence of the highest concentrations of the peptides, ca. 80% of the triplets of 4CB was quenched (30% for enkephalins).

Reference spectra for the 4CB triplet, the ketyl radical ($CBH^{\bullet}$) and the ketyl radical anion ($CB^{\bullet -}$) used in the spectral deconvolutions were obtained following the laser flash photolysis of 4×10^{-3} M aqueous solutions of 4CB at pH 10.0, pH 6.5 and pH 10.5, respectively. The two latter solutions contained additionally 1.3 M 2-propanol (isopropanol, *i*PrOH) serving as a hydrogen-atom donor. Spectra of the tyrosyl radical ($TyrO^{\bullet}$) and the $(S_{\cdot}{}^{\cdot}N)^+$ radical cation came from pulse radiolysis experiments and were kindly provided by Prof. K. Bobrowski from the Institute of Nuclear Chemistry and Technology, Warsaw, Poland. Tyrosyl radicals were generated by pulse-irradiating N_2O-saturated neutral aqueous solutions of 2×10^{-3} M tyrosine containing 0.1 M NaN_3. The spectrum of the $(S_{\cdot}{}^{\cdot}N)^+$ transient was obtained by pulse-irradiating N_2O-saturated aqueous solutions of 1×10^{-4} M MetGly at pH 5.

3. Results and discussion

In order to elucidate the participation of the Tyr and/or the Met residue in the photooxidation of the MetEnk, three groups of model peptides were used as 4-carboxybenzophenone triplet quenchers: (i) peptides containing only tyrosine (Tyr, TyrGly, TyrGlyGly, LeuEnk), (ii) peptides containing only Met or no tyrosine (Phe, PheMet) and (iii) peptides containing both amino acid residues (TyrMet, MetTyr, MetEnk). In the presence of the investigated peptides, an accelerated decay of the 4CB triplet and formation of new light-absorbing transients were observed. Qualitative and quantitative analysis of these data were performed.

3.1. Quenching rate constants

In order to determine the quenching rate constants (k_q), the decay of the triplet state of 4CB (^{3}(4CB)*) was monitored at 450 and 540 nm in the presence of increasing concentrations of the quenchers. The triplet state of 4CB exhibits an absorption maximum at 540 nm. However, its absorption in this spectral range overlaps with the absorption of the electron-transfer intermediates, i.e. the ketyl radical $CBH^{\bullet}$ and the ketyl radical anion $CB^{\bullet -}$, both of which can be formed in the quenching process [23,24]. Therefore, the latter wavelength, 450 nm, is more appropriate for the kinetic studies of the triplet.

All of the quenching experiments were carried out at neutral pH, therefore peptides were in their zwitterionic forms, and 4CB had a negative charge (pK_a (4CB) = 4.5 [25,26]). Under our experimental conditions, it was possible to fit all the time profiles with first-order exponential decay functions in order to estimate the acceleration of the triplet decay (shortening of the triplet lifetime τ) with increasing quencher concentrations. From the slope of the Stern-Volmer type plots, representing changes of the observed decay rate constants ($k_{obs} = 1/\tau$) vs. concentrations of the investigated peptides, the quenching rate constants k_q were determined (Table 1). An example of the triplet decay at 450 nm in the presence of the increasing concentrations of the quencher, together with the Stern-Volmer type plot based on it, is shown in Fig. 1.

Table 1
Rate constants for quenching of the 4CB triplet by MetEnk and its model peptides

Quencher Q	pK_a	pH[a]	$z_Q \times z_{4CB}$[b]	$k_q \times 10^{-9}$ (M^{-1} s^{-1})[c]
Tyr	2.2; 9.1; 10.1[e]	7.3	0	1.3
TyrGly	3.13; 7.54; 9.86[f]	7.0	0	1.3
TyrGlyGly	3.19; 7.37; 10.09[g]	7.0	0	1.3
Met	2.3; 9.2[e]	6.8	0	2.2[d]
Phe	2.6; 9.2[e]	5.5	0	0.1
PheMet	3.24; 7.27[h]	6.9	0	1.7
TyrMet	–[i]	6.9	0	2.4
MetTyr	3.19; 7.36[j]	6.5	0	3.0
TyrGlyGlyPheMet	3.46; 7.44; 9.82[k]	7.2	0	1.9
TyrGlyGlyPheLeu	7.28; 9.73[l]	6.4	0	1.4

[a] No buffer.
[b] Net charge on the quencher (z_Q) multiplied by net charge on the photosensitizer (z_{4CB}).
[c] Estimated error ±20%.
[d] From Ref. [14].
[e] Ref. [45].
[f] Ref. [46].
[g] Ref [47].
[h] Ref. [48].
[i] No information available. pK_a values presumably like for MetEnk.
[j] Ref. [49].
[k] Ref. [50].
[l] Data for LeuEnk amide; see Ref. [51]; pK_a value of carboxylic group like for MetEnk.

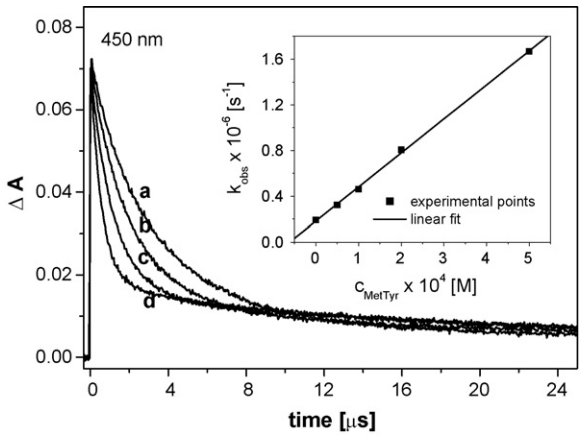


Fig. 1. Decays of the 4CB triplet state generated in laser flash photolysis of the neutral aqueous solutions of 4×10^{-3} M 4-carboxybenzophenone in the presence of the increasing concentrations of MetTyr (excitation wavelength: 355 nm, laser energy: 3 mJ) (a) 5×10^{-5} M; (b) 1×10^{-4} M; (c) 2×10^{-4} M; (d) 5×10^{-4} M MetTyr inset: Stern-Volmer type plot for quenching of the 4CB triplet by MetTyr.

All the determined quenching rate constants are close to the diffusion-controlled limit, which indicates that electron transfer is a primary step of the triplet quenching. For peptides containing only tyrosine, the obtained k_q values are ca. 1.3×10^9 M^{-1} s^{-1}, whereas for all the methionine-containing peptides, the k_q values are higher: ca. $(1.7–1.9) \times 10^9$ M^{-1} s^{-1} for PheMet and MetEnk, 2.4×10^9 M^{-1} s^{-1} for TyrMet and 3.0×10^9 M^{-1} s^{-1} for MetTyr. These data indicate slightly higher reactivity of the Met-residue towards the triplet of 4CB when compared to the Tyr-residue. Since the triplet quenching rate constants (k_q) determined for the single amino acids may to some extend differ from the k_q values for the same amino acid residues when they are located in a single peptide, no clear quantitative conclusion can be made with respect to the ratio of Met to Tyr undergoing 4CB sensitized oxidation when both amino acids are located in a single molecule. Therefore, it is reasonable to assume that statistically 50% of Tyr and 50% of Met in the MetEnk molecules react with the photosensitizer, since even if the Met-residue indeed reacts slightly faster with the 4CB triplet than the Tyr-residue, i.e. less than 50% of Tyr undergoes oxidation, our considerations about the mechanism of the MetEnk photooxidation still remain valid. Contribution of the phenylalanine to the photosensitized oxidation of MetEnk can be neglected taking into account the low reactivity of Phe towards the triplet of 4CB when compared to all the other amino acids and peptides studied.

The differences in the triplet quenching rate constants for tyrosine and methionine result from the differences in their oxidation potentials. Therefore, comparison of the oxidation potentials of these amino acids should enable to estimate the ratio between Tyr and Met reacting with the photosensitizer. However, the oxidation of Met in water solutions is an irreversible process. Therefore, only the peak anodic oxidation potential E_p (Met/Met$^{\bullet+}$) has been reported for methionine (1.3 V and 1.2 V vs. SCE at pH 8.2 and pH 12.2, respectively; [27]), which can be treated as the upper limit of the standard reduction potential E_0 (Met/Met$^{\bullet+}$) for this amino acid. In acetonitrile, value of 1.1 V vs. SCE was estimated as the Met oxidation potential [28]. However, also in this solvent, oxidation reaction of methionine was irreversible. As documented for tyrosine in aqueous solutions, its oxidation potential equals to 1.2 V and 0.5 V vs. SCE at pH < 10.5 and pH > 10.5, respectively [13]. Based on the reported oxidation potentials of Tyr and Met, only general conclusion about very similar reactivity of these amino acid residues in the triplet quenching can be made. Therefore, triplet quenching rate constants seem to be more reliable parameter to estimate the ratio between Tyr and Met undergoing photooxidation.

3.2. Resolution of the transient spectra

As mentioned in the previous paragraph, addition of the quencher not only accelerates decay of the triplet, but also results in the formation of new absorption bands. The absorption maximum observed at 570 nm was attributed to the 4CB ketyl radical, and the absorption maximum at 660 nm was attributed to the 4CB ketyl radical anion. Both of these species were easily detectable due to their high molar absorption coefficients (ε = 5200 and 7660 M^{-1} cm^{-1}, respectively) [23,24]. Unlike these electron-transfer intermediates derived from the photosensitizer, the products of the oxidation of the tyrosine and methionine residues were more difficult to detect.

Tyrosyl radicals exhibit a sharp optical absorption band at λ = 405–410 nm with a relatively low molar absorption coefficient of ca. 2750 M^{-1} cm^{-1} [29,30] or 3200 M^{-1} cm^{-1} as reported by Bansal and Fessenden [31]. Hence, their weak absorption under our experimental conditions was masked by the absorption of other transients. However, in the presence of oxygen, when Tyr-containing peptides at concentration of 0.5×10^{-3} M were used as quenchers, the absorption spectrum of the TyrO$^{\bullet}$ radicals was registered on the long-time scale. Tyrosyl radicals are practically unreactive towards oxygen ($k < 1 \times 10^3$ M^{-1} s^{-1}) [32], whereas the 4CB triplet, the CBH$^{\bullet}$ radical and the CB$^{\bullet-}$ radical anions react with oxygen at a diffusion-controlled rate [33]. Therefore, these latter species were immediately quenched in the presence of oxygen.

Sulfur-centered radical cations cannot be detected by optical absorption, unless stabilised by two-center three-electron bonds, e.g., involving nitrogen ((S∴N)$^+$, λ_{max} = 380 nm), sulfur ((S∴S)$^+$, λ_{max} = 480–490 nm) [14], or oxygen ((S∴O), λ_{max} = 390 nm) [34]. None of these species was directly observed in our systems.

Although no products of sulfur photooxidation were detected, their existence could not be excluded solely on the basis of standard transient spectra analysis because of the strong absorptions of the ketyl radicals and the ketyl radical anions of 4CB which dominate in the spectral range of interest. In addition, the 4CB triplet was not always completely quenched because of the limited solubility of the quenchers. As a consequence, no quantitative analysis could be carried out. Therefore, a technique of resolving the time-dependent transient spectra into their spectral components was applied. This resolution method is analogous to a linear regression technique of the form:

$$\Delta A(\lambda_j) = \sum_{i=1}^{n} \varepsilon_i(\lambda_j) c_i l \qquad (1)$$

where $\Delta A(\lambda_j)$ is the observed absorbance change of the composite spectrum at the jth wavelength, $\varepsilon_i(\lambda_j)$ is the molar absorption coefficient of the ith species at the jth wavelength of the observation, c_i is the concentration of the ith transient, and l is the optical path length. When molar absorption coefficients of all possible transients present in the system are known at different wavelengths, a set of equations can be solved, in a least-squares fashion, to give the unknown concentrations of the intermediates, c_i. Once these concentrations are determined, the transient spectra are normalized to the ΔA corresponding to their absorption in the composite spectrum. This enabled us to extract the absorption spectra of transient species that are partially hidden by the absorption of other transients and to obtain concentration profiles of the overlapping intermediates. Further details of this resolution method have been described elsewhere [28].

Such a spectral resolution requires isolated reference spectra of all likely transients, together with their molar absorption coefficients $\varepsilon_i(\lambda_j)$. The reference spectra were obtained in laser flash photolysis and pulse radiolysis experiments, as described in Section 2. Reference spectra of the ^{3}(4CB)*, CBH$^\bullet$, and CB$^{\bullet-}$ were normalized to the molar absorption coefficients used earlier by Marciniak et al. [23] The reference spectrum of the tyrosyl radical was normalized to the molar absorption coefficient equal to 2750 M^{-1} cm^{-1} [29,30], which was considered to be most reliable. The presence of the intermolecularly bonded $(S_{\cdot}{\cdot}S)^+$ species was excluded due to low concentrations of the quenchers (well below 2×10^{-2} M) [14,16]. Based on the literature data, the formation of the $(S_{\cdot}{\cdot}N)^+$ transient needed to be taken into account only when Met was at the N-terminus of the peptide [35]. Since no $(S_{\cdot}{\cdot}O)$ species has been detected in the experimental systems analogous to the one investigated by us in this paper, the possible existence of this species was neglected in our considerations [14].

Spectral resolution was performed on composite transient spectra taken at different time-delays registered in the presence of the quenchers allowing ca. 30% triplet quenching for enkephalins and ca. 80% triplet quenching for all the other amino acids and peptides, as dictated by their solubility. By including the reference spectra of all the previously mentioned transients and the spectrum of the 4CB triplet, satisfactory resolutions of all the composite spectra were obtained (Fig. 2). The highest deviations in the composite fits of the experimental data were usually observed in the spectral range of 390–410 nm, i.e. where the sharp absorption band of the tyrosyl radicals was strongly masked by the absorption of ^{3}(4CB)*, ketyl radicals, and ketyl radical anions of 4CB. There was additional uncertainty in the calculated concentrations of the tyrosyl radicals which was due to the lack of agreement in the molar absorption coefficient of TyrO$^\bullet$ reported in the literature [29–31]. These uncertainties are reflected in the values of the calculated quantum yields of TyrO$^\bullet$ (cf. Table 1), some of which are higher than 1, but obviously should be considered as not higher than this value.

When MetTyr was used as the triplet quencher at neutral pH, it was necessary to include the reference spectrum of the $(S_{\cdot}{\cdot}N)^+$ species in order to obtain good agreement between the composite fits and the experimental spectra. However, at alkaline pH good fits were obtained without including this intermediate. The latter case can be explained by the much faster decay of the $(S_{\cdot}{\cdot}N)^+$ transient at higher pH [14,36]. The presence of this species at neutral pH, where normally the free amino group of the investigated dipeptide is protonated which disables formation of the intramolecular $(S_{\cdot}{\cdot}N)^+$ bond, can be connected with an additional proton transfer reaction from the protonated amino group to the ketyl radical anion within radical-ion pair [17] or with presence of some free amino groups even at neutral pH.

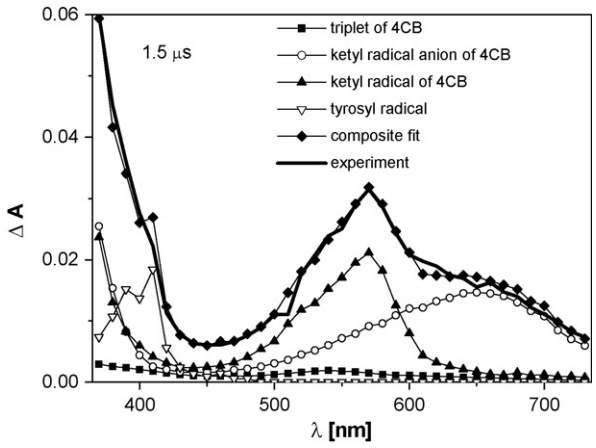


Fig. 2. Typical spectral resolution of the composite transient spectrum formed in the laser flash photolysis of aqueous solution of 4×10^{-3} M 4-carboxybenzophenone and 5×10^{-4} M TyrMet at pH 6.9 (excitation wavelength: 355 nm; laser energy: 3 mJ) taken 1.5 μs after the laser pulse.

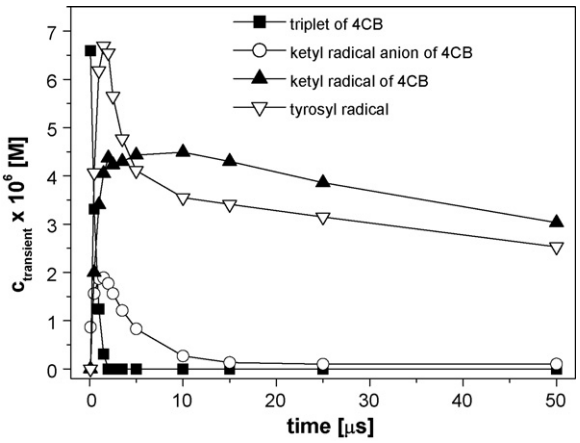


Fig. 3. Concentration profiles of the transient species formed in the laser flash photolysis of aqueous solution of 4×10^{-3} M 4-carboxybenzophenone and 5×10^{-4} M TyrMet at pH 6.9 (excitation wavelength: 355 nm; laser energy: 3 mJ). The concentrations were determined by spectral deconvolution method.

Concentrations of the separate transients obtained from the spectral-resolution procedure were subsequently plotted as a function of time (Fig. 3). As explained in the above paragraph, the error of the determined transients' concentrations was highest for the tyrosyl radicals. The relatively long lifetime of the triplet in the investigated systems and the rapid establishment of the acid–base equilibrium between CBH$^\bullet$ and CB$^{\bullet-}$ did not allow us to derive precise concentrations for the CBH$^\bullet$ and CB$^{\bullet-}$ species formed just after the quenching event. Only the overall concentration of both species present in the system when the 4CB triplet was totally quenched could be precisely determined. The concentration of the TyrO$^\bullet$ radicals reached a maximum when the triplet of 4CB was fully quenched. Subsequently, the decay of the tyrosyl radicals and slow decays of the ketyl radicals and ketyl radical anions of 4CB were observed. The ratio between ketyl radicals and ketyl radical anions of 4CB varied depending on pH, with the latter species prevailing in alkaline solutions (pK_a (CBH$^\bullet$) = 8.2 [25]). No secondary growth in the concentrations of the 4CB-derived electron-transfer intermediates was observed.

3.3. Quantum yields

Quantum yields of the transients were determined using a relative actinometry method. Samples with equal concentrations of 4CB were prepared. Those containing no quencher were used as the external actinometer, whereas to all other samples quenchers were added. Quantum yields were calculated according to a following formula:

$$\Phi = \frac{c\varepsilon_T l}{\Delta A_T^0} \quad (2)$$

where c is the concentration of the transient as determined from the concentration profile obtained in spectral deconvolutions, ε_T is the molar absorption coefficient of the 4CB triplet, l is the optical path length, and ΔA_T^0 is the absorption change in the actinometer immediately after the laser pulse at 540 nm, i.e. at the maximum of the triplet absorption. Concentrations of the transients were taken from the concentration profiles at a point in time when the triplet

Table 2
Quantum yields of the transients for the 4CB-sensitized photooxidation of MetEnk and its model peptides

Quencher	pH[a]	$\Phi_{CBH^{\bullet}+CB^{\bullet-}}$ [b]	$\Phi_{TyrO^{\bullet}}$ [b]	$\Phi_{SN^{+}}$ [b]
Tyr	7.3	0.91	1.13[c]	–
TyrGly	7.0	0.99	1.22[c]	–
TyrGlyGly	7.0	0.83	0.88	–
Met[d]	6.0	0.38	–	0
PheMet	6.9	0.35	–	–
TyrMet	6.9	0.60	0.66	–
	10.2	0.68	0.69	–
MetTyr	6.5	0.74	0.71	0.09
	10.2	0.77	0.79	–
TyrGlyGlyPheMet	7.2	0.82	0.81	–
	10.5	0.75	0.82	–
TyrGlyGlyPheLeu	6.0	1.13	1.06	–
	10.2	1.00	1.05	–

[a] No buffer.
[b] Estimated error ±20%.
[c] Quantum yields of the tyrosyl radicals higher than 1 are due to the factors discussed in text (cf. Section 3.2) and have to be considered as not higher than 1.
[d] From Ref [15].

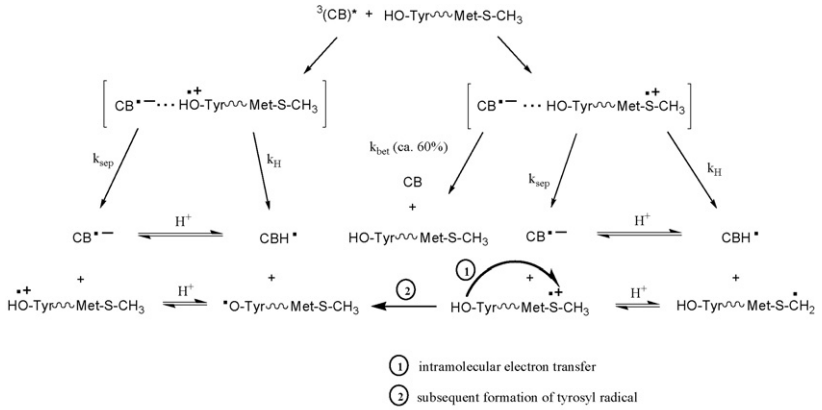
state had already decayed in the system. Taking into account only 30% triplet quenching by the enkephalins and 80% by other amino acids and peptides, an additional correction for the full quenching of the triplet was made. The values of the quantum yields determined in this manner are given in Table 2.

For the quenchers containing only the tyrosine residue (Tyr, TyrGly, TyrGlyGly and LeuEnk), the quantum yield of tyrosyl radicals equals the sum of the quantum yields of the ketyl radicals and ketyl radical anions of 4CB. Both of these quantum yields are close to unity. Therefore, the radical-ion pair [$4CB^{\bullet-}\cdots Q^{\bullet+}$] formed in the reaction of the 4CB triplet with the tyrosine-containing quencher must decay by separation of the radical ions and/or by proton transfer within ion-pair and subsequent separation of the neutral radicals (cf. Section 1 and Scheme 2). The ratio of these two processes could not be estimated because of the immediate deprotonation of the tyrosine radical cation. When only methionine is present in the quencher molecule, the sum of the quantum yields of $CBH^{\bullet}$ and $CB^{\bullet-}$ equals ca. 0.4, which indicates that the back electron-transfer process contributes up to 60% to the decay of the radical-ion pair [$4CB^{\bullet-}\cdots Q^{\bullet+}$] (cf. Section 1 and Scheme 2). When both the tyrosine and the methionine residues are present in the quencher molecule (TyrMet, MetTyr, MetEnk), the yields of the tyrosyl radicals decrease to the value of approximately 0.7–0.8, which equals to the sum of the quantum yields of the ketyl radicals and ketyl radical anions of 4CB. The above observations are discussed further below.

3.4. Reaction mechanism

The above observations can be explained in terms of the competitive oxidation reactions involving Tyr- and Met-residues followed by the intramolecular electron transfer from the tyrosine residue to the sulfur-centered radical cation on the methionine residue. Under our experimental conditions, intermolecular electron transfer can be neglected. Since it is a diffusion-controlled process, it can compete with intramolecular electron transfer only when higher concentrations of peptides are used. The mechanism describing the processes involved in the quenching of the 4CB triplet by Met-enkephalin and other peptides, containing both Tyr- and Met-residues, is given in Scheme 2.

As far as the photooxidation of the methionine residue is concerned, this scheme is analogous to the one previously reported for sulfur-containing amino acids, Met-containing dipeptides and tripeptides and methionine derivatives [16,35,37]. Statistically, 50%



k_{sep} rate constant for the charge separation
k_H rate constant for the proton transfer within a radical-ion pair followed by separation of the radicals
k_{bet} rate constant for the back electron-transfer

Scheme 2. Quenching of the 4CB triplet state by MetEnk.

of Tyr and 50% of Met in the MetEnk molecules undergo photooxidation while reacting with the triplet state of 4CB. Therefore, the primary step of the triplet quenching involves the formation of two different radical-ion pairs [4CB$^{\bullet-}\cdots$pep-(Met)S$^{\bullet+}$] and [4CB$^{\bullet-}\cdots{}^{+\bullet}$O(Tyr)-pep], with the assumed yield of 0.5 each. From [4CB$^{\bullet-}\cdots{}^{+\bullet}$O(Tyr)-pep], tyrosyl radicals ($\Phi_{\mathrm{TyrO}\bullet} = 0.5$) as well as ketyl radicals and ketyl radical anions of 4CB ($\Phi'_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}} = 0.5$) are formed. Simultaneously, from [4CB$^{\bullet-}\cdots$pep-(Met)S$^{\bullet+}$], sulfur-centered radical cations as well as ketyl radicals and ketyl radical anions of 4CB are generated. Due to the back electron-transfer process (60%), the sum of the quantum yields of CBH$^{\bullet}$ and CB$^{\bullet-}$ formed in this channel ($\Phi''_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}}$) is decreased to the value of 0.2, which has to equal the quantum yield of the sulfur-centered radical cations ($\Phi_{\mathrm{S}^{\bullet+}}$, not detected directly by the optical absorption). Therefore, the overall yield of CBH$^{\bullet}$ and CB$^{\bullet-}$ ($\Phi_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}} = \Phi'_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}} + \Phi''_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}}$) must be equal to ca. 0.7. If no other process occurred in the system, the quantum yield of tyrosyl radicals should always be lower than $\Phi_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}}$ and equal to ca. 0.5, which is not observed. Yet, for all the quenchers containing both Tyr- and Met-residues, $\Phi_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}} = \Phi_{\mathrm{TyrO}\bullet} \approx 0.7 - 0.8$. This indicates ca. 100% efficient intramolecular electron transfer from the tyrosine to the sulfur-centered radical cation of the methionine residue, which leads to the increase of $\Phi_{\mathrm{TyrO}\bullet}$ by ca. 0.2 ($\Phi_{\mathrm{TyrO}\bullet} = 0.5 + \Phi_{\mathrm{S}^{\bullet+}} \approx 0.7$). It does not influence, however, the value of $\Phi_{\mathrm{CBH}^{\bullet+}\mathrm{CB}^{\bullet-}}$. All quantum yields estimated in such a manner are in very good agreement with the experimentally determined values.

According to Scheme 2, the postulated intramolecular electron transfer should compete with deprotonation of the sulfur-centered radical cations, a process which would produce α-thio-alkyl radicals. These radicals could also be formed due to a proton transfer within the [4CB$^{\bullet-}\cdots$pep-(Met)S$^{\bullet+}$] radical-ion pair, followed by a further separation of the neutral radicals. However, based on the quantum yields determined, we may assume that both of these processes are negligible in our system.

The question arises whether the observed intramolecular electron transfer occurs through space (solvent) or through the peptide bonds. The difficulty in elucidating this is twofold. One complication is the large variety of conformations in which the enkephalins can exist in aqueous solutions. The other complication is the rate at which the enkephalins can fold, i.e. flip between folded and extended conformation, with respect to the rate of the intramolecular electron transfer. In the solid state MetEnk is postulated [38] to be only in an extended structure, but, in solution, molecular dynamics simulations, theoretical conformational analysis, and NMR studies indicate the existence of an ensemble of folded forms, being always in equilibrium with the extended conformation [39–42]. With regard to the folded conformations, only the proximity of the Tyr^1 and Phe^4 has been reported [41]. However, there is no information on the distance between the tyrosine aromatic ring and the aliphatic side chain of methionine. From early NMR studies, it is known that the MetEnk conformation is a function of pH. In particular, MetEnk has at least two different conformations, one at low and one at high pH, and these conformations are characterized by the folding of the molecule [43]. A more recent Raman spectral analysis suggests a conformational equilibrium rather than a preferred conformation in a given pH range. Nevertheless, one may state that at higher pH (pH > 9) the extended conformation prevails due to the repulsive electrostatic forces between the ionized carboxylic group and the ionized OH group of Tyr [44]. Similarly, at neutral pH, folded conformations tend to dominate due to the electrostatic interaction between the protonated amino group at the N-terminus and the deprotonated C-terminal carboxylic group. Taking all these considerations into account, we suspect that most probably in our system an intramolecular electron transfer occurs via peptide bonds since, regardless of pH, we observe the same quantum yields for all the transients.

4. Conclusions

Quenching of the 4CB triplet state by methionine-enkephalin and its model peptides was studied by means of laser flash photolysis. The primary step of triplet quenching involved an electron transfer and the formation of a radical-ion pair, as confirmed by large quenching rate constants and the existence of radical-ion intermediates. When both, Met- and Tyr-residues were present in the molecule, a competitive photooxidation took place. Statistically, 50% of Met and 50% of Tyr in the MetEnk molecules served as the electron donors for the 4CB triplet state. The subsequent fate of the radical-ion pair was characterized by determining the quantum yields of the electron-transfer intermediates, namely the ketyl radical CBH$^{\bullet}$, the ketyl radical anion CB$^{\bullet-}$, the tyrosyl radical TyrO$^{\bullet}$, and the $(\mathrm{S}_{\therefore}\mathrm{N})^{+}$ radical cation. The overlapping transient spectra were resolved into single spectral components, and, therefore, it was possible to monitor the kinetic behavior of all of the species present in the investigated system after the triplet-quenching event. On the basis of the quenching rate constants and the observed quantum yields, a reaction mechanism is proposed. In this mechanism, an intramolecular electron transfer from the tyrosine residue to the sulfur-centered radical cation on the Met-residue takes place with 100% efficiency. This electron-transfer process is postulated to occur via peptide bonds.

Acknowledgements

This work was supported by the European Research Training Network SULFRAD (contract number: HPRN-CT-2002-00184) as well as the Marie-Curie European Reintegration Grant (contract number: MERG-CT-2006-031402).

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