ELSEVIER

Biophysical Chemistry 92 (2001) 119–126

Biophysical Chemistry

www.elsevier.com/locate/bpc

Protection of radiation-induced protein damage by curcumin

S. Kapoor*, K.I. Priyadarsini

Radiation Chemistry and Chemical Dynamics Division, Bhabha Atomic Research Center, Mumbai 400 085, India

Received 19 March 2001; received in revised form 25 June 2001; accepted 25 June 2001

Abstract

Free radical reactions of lysozyme (Lz), tryptophan and disulfides were studied with curcumin, a lipid-soluble antioxidant from turmeric, in aqueous solution using a pulse radiolysis technique. The binding of curcumin with lysozyme was confirmed using absorption, fluorescence and stopped-flow techniques. The free radicals of curcumin generated after repairing radicals of disulfides, lysozyme and tryptophan absorb at 500–510 nm. Implication of this in evaluating the antioxidant behavior of curcumin in protecting proteins is discussed.

Keywords: Lysozyme; Electron transfer; Pulse radiolysis, Antioxidant; Curcumin; Tryptophan radical

1. Introduction

Radiation damage to proteins has been the subject of great attention because of the current interest in radiobiology and radio sterilization of food and enzymes [1]. Several studies have demonstrated that reduction/oxidation reactions in proteins are intimately involved in the control of vital processes such as signal tranduction, gene expression etc. [2]. While the oxidation takes place at the amino acid site, the reduction is mostly observed on the disulfide bond. Some reports have also indicated that the fragmentation of peptides, polypeptides and proteins in the solid state involves the dry electron [3]. Long-range electron transfer between the phenol side chain of tyrosine and tryptophan indolyl radical has been observed in a number of proteins and also in model systems separated by peptide bridges [4–13]. It has been suggested that such long-range electron transfer may extend the target size for radiation-induced damage of the sub-cellular pro-

* Corresponding author. Fax: +91-22-5505151.
E-mail address: sudhirk@apsara.barc.ernet.in (S. Kapoor).

0301-4622/01/$ - see front matter
PII: S0301-4622(01)00188-0

tein. Also, some studies indicate that the hole center produced mainly by the charge transfer from ionized solvent molecules can get localized at the amide back bone [14].

The oxidation and reduction of proteins are often mediated by free radicals produced either due to a metabolic process or during oxidative stress. Antioxidants are known to protect cells and also proteins from the free radical attack. In recent years, it is a matter of great interest that dietary antioxidants can decrease the risk of many diseases [15–17]. Turmeric (*Curcuma longa)* is one such natural antioxidant, which is used as an ingredient in Indian cooking. The major yellow pigment of turmeric is curcumin, a polyphenol. Like vitamin E (α-tocopherol), curcumin is a lipid-soluble antioxidant and is soluble only in organic solvents and membranes. Very recently, free radical reactions and chemo-preventive mechanisms of curcumin have been reported [18–23]. In view of the importance of dietary antioxidants, we have studied the free radical reactions of curcumin with a protein, lysozyme (Lz) and some model compounds that are the constituents of lysozyme.

2. Experimental

2.1. Materials

Curcumin, lysozyme, tryptophan, cystine, obtained from Sigma, were used as received. All other chemicals used were either AR or GR grade. Nanopure water was used throughout the experiments. IOLAR grade gases (purity ≥ 99.9%) were used for purging the solutions. All solutions were prepared just before the experiments and kept in dark to avoid any photochemical reactions.

2.2. Methods

2.2.1. Absorption, fluorescence and stopped-flow techniques

Steady-state fluorescence and absorption measurements were carried out using Hitachi (F-4010) spectrofluorimeter and a Hitachi (330) spectrophotometer, respectively. The fluorescence anisotropy experiments were carried out both in the presence and the absence of lysozyme and the details are given as follows. The solutions with and without lysozyme (1 mM) containing curcumin (22 μM) and 2% methanol were excited at wavelength 428 nm. The polarized emission in parallel and perpendicular direction was collected in the wavelength region 500–600 nm. The steady-state anisotropy $\langle r \rangle$ is defined as:

$$\langle r \rangle = (I_{\parallel} - I_{\acute{A}})/((I_{\parallel} + 2I_{\acute{A}}) \quad (1)$$

where $I_{\parallel}$ and $I_{\acute{A}}$ are, respectively, the polarized fluorescence intensities parallel and perpendicular to the excitation polarization. I_{II} and $I_{\acute{A}}$ were corrected for the different sensitivity depending on polarization.

A stopped-flow spectrometer (SX-18MV, Applied Photo physics Ltd., UK) with absorption detection was employed to study the binding constant of lysozyme with protein. In brief, equal volumes of two solutions containing curcumin in methanol/water mixture (1:99 v/v) and aqueous solutions of lysozyme were mixed simultaneously with the help of a pneumatic drive and the changes in curcumin absorption were monitored with absorption detection. The kinetic data was fitted to a single exponential function. At least three different runs were used to get the observed rate constant.

2.2.2. Pulse radiolysis

7 MeV electron pulses of 50 ns from a linear electron accelerator (LINAC) were employed for pulse radiolysis studies. The details of LINAC are given elsewhere [24]. When short pulses of high-energy electrons interact with water, the energy deposited results in the formation of primary radicals viz. e^-_{aq}, $OH^{\cdot}$ and $H^{\cdot}$. One can selectively scavenge the $OH^{\cdot}$ radicals or e^-_{aq} to study oxidation and reduction reactions. The $OH^{\cdot}$ radicals formed by the water radiolysis and also by Eq. (2) react rapidly with N_3^- (0.1 M) and produce azide radicals Eq. (3), which are specific one-electron oxidant with the reduction potential of 1.33 V vs. NHE [25].

$$e_{aq}^- + N_2O + H_2O \rightarrow OH^{\cdot} + N_2 + OH^- \quad (2)$$

$$OH^{\cdot} + N_3^- \rightarrow OH^- + N_3^{\cdot} \quad (3)$$

The reduction of disulfides was studied in N_2-bubbled aqueous solutions containing 0.1 M *t*-butanol. The transients formed after reactions with primary and secondary radicals were monitored using kinetic spectrophotometry. The absorbed dose was measured using aerated thiocynate dosimeter solution. All the experiments were carried out at pH 6.8 using equimolar mixture of phosphates (5 mM) as buffers.

3. Results and discussion

3.1. Binding of curcumin to lysozyme

Curcumin being lypophillic gets solubilized into the hydrophobic pockets of the proteins. Therefore, it should be possible to observe changes in absorption and fluorescence spectra of curcumin in the presence of lysozymes as the absorption and fluorescence properties of curcumin are very sensitive to the solvent environment [26]. Curcumin is insoluble in water and hence it is necessary to add a minimum of 1% methanol to solubilize at least 20–50 μM curcumin. Curcumin exhibits intense absorption in the wavelength range from 300 to 500 nm with maximum absorption at 420 nm. Lysozymes do not absorb above 300 nm and therefore do not interfere with the absorption spectrum of curcumin. However, when lysozyme (1 mM) solution was added to curcumin (22 μM) the shape of the absorption spectrum did not change but the absorbance at 420 nm increased with increasing lysozyme concentration. Fig. 1 shows the absorption spectrum of curcumin in the presence of varying amounts of lysozyme, suggesting existence of equilibrium between curcumin and lysozyme. Therefore, it is possible to apply Benesi–Hilderbrand equation [27] for such equilibrium [Eq. (4)]:

$$\text{lysozyme} + \text{curcumin} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{lysozyme-curcumin} \quad (4)$$

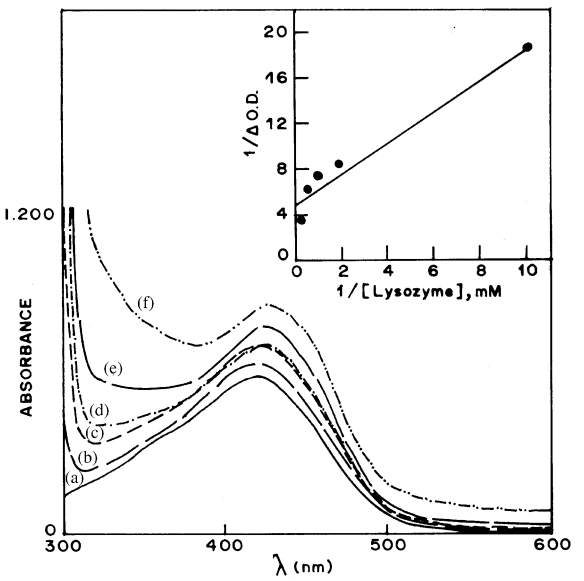

Fig. 1. Absorption spectra of 22 μM curcumin in the presence of various concentrations of lysozyme. (a) [Lz] = 0.0 M, (b) [Lz] = 1.0×10^{-4} M, (c) [Lz] = 5.0×10^{-4} M, (d) [Lz] = 1.0×10^{-3} M, (e) [Lz] = 2.0×10^{-3} M and (f) [Lz] = 5.0×10^{-3} M. All solutions contained 1% methanol. Inset shows linear plot for the reciprocal of absorbance increase at 420 nm of reciprocal concentration of lysozyme.

Assuming a 1:1 complex and using Benesi–Hilderbrand equation in terms of reciprocal OD at 420 nm as a function of reciprocal concentration of lysozyme [27], a linear plot is obtained as given in the inset of Fig. 1. From this, an equilibrium constant of 1230 M^{-1} was estimated between curcumin and lysozyme.

Like absorbance, the fluorescence intensity from the excited state of curcumin at 510 nm (Fig. 2) was found to increase in the presence of lysozyme. The rate of increase in fluorescence was found to be more than that of absorption. Hence, the changes in fluorescence can be utilized to evaluate binding constant with suitable corrections. However, no such attempt was made in the present work.

The binding constant was also determined by using the stopped-flow technique. Since there is a change in the absorbance in the presence of lysozyme, the rate of change in the absorbance due to curcumin is followed as a function of time,

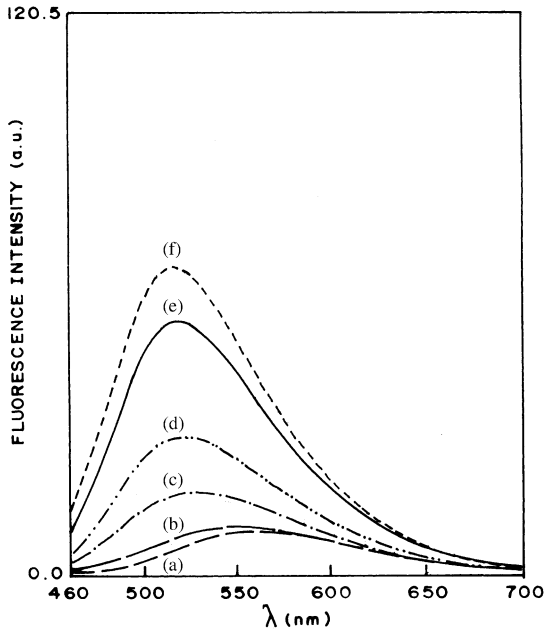

Fig. 2. Fluorescence spectra of 22 μM curcumin in the presence of various concentrations of lysozyme. (a) [Lz] = 0.0 M, (b) [Lz] = 1.0×10^{-4} M, (c) [Lz] = 5.0×10^{-4} M, (d) [Lz] = 1.0×10^{-3} M, (e) [Lz] = 2.0×10^{-3} M and (f) [Lz] = 5.0×10^{-3} M. λ_{exc} = 428 nm. All solutions contained 1% methanol.

at a given concentration of lysozyme. Inset of Fig. 3 shows a typical absorption–time plot, indicating increase in absorbance at 420 nm with time after mixing 25 μM solution of curcumin in 1% methanol with a solution of 1 mM lysozyme. The observed rate constant (k_{obs}) was found to increase with increasing concentration of lysozyme from 0.6 to 4 mM. Thus, for the equilibrium between curcumin and lysozyme as given in Eq. (4), the binding constant can be related to k_{obs} in terms of the forward (k_f) and reverse (k_b) rate constants by Eq. (5):

$$k_{obs} = k_f[\text{lysozyme}] + k_b \quad (5)$$

From the slope and intercept of the linear plot of the observed rate constant (k_{obs}) vs. lysozyme concentration (Fig. 3), the binding constant (k_f/k_b), was calculated to be 875 M^{-1}. This further supports our earlier speculation that curcumin has selective affinity towards protein by solubilizing in the hydrophobic micro domains of the protein.

The discrepancy observed in the binding constant of curcumin with lysozyme obtained from steady-state absorption method and utilizing stopped-flow technique can be explained as follows. The steady-state absorption method considers the overall increase in the absorbance and does not account for any free curcumin present in the solution. Whereas, the time-resolved method considers only the bound curcumin, hence may be more reliable than the steady-state absorbance method.

To reconfirm the binding of curcumin with lysozyme, fluorescence anisotropy experiments were also carried out. The steady-state anisotropy values ($\langle r \rangle$) for the solution containing 1 mM lysozyme and 22 μM curcumin were found to be in the range 0.33–0.36. The results are shown in Fig. 4. It can be seen from the same figure that fluorescence anisotropy of curcumin in the absence of lysozyme is negligibly small (Fig. 4a). This shows that anisotropy of curcumin increases significantly in the presence of 1 mM lysozyme and was independent of the wavelength (Fig. 4b). This further confirms that curcumin gets solubilized in the hydrophobic micro domains of lysozyme, where it experiences a restricted motion and therefore, an increase in the anisotropy occurs. It is important to mention here that the lifetime of curcumin varies from 200 to 400 ps in various polar solvents [26], hence, the probability of polarization is more. All these observations and a large binding constant observed by following the changes in the absorption of curcumin suggest that curcumin binds to lysozyme irreversibly.

3.2. Pulse radiolysis studies

If curcumin binds to the proteins, it should also be possible to protect proteins from free radical-induced damage. Free-radical-induced oxidation of proteins has been found to be mainly on the amino acid site and oxidized tryptophan and tyrosine radicals have been observed during protein oxidation. Antioxidants, capable of scavenging the free radicals can be employed to minimize the

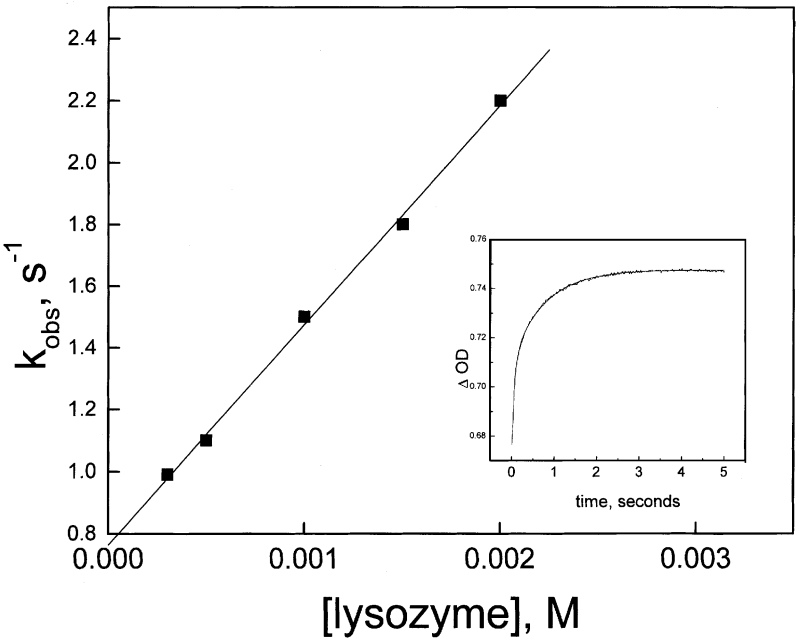

Fig. 3. Plot showing the variation in the observed binding rate constant with the concentration of lysozyme. Inset shows absorption–time plot for binding of 23 μM curcumin in 1% methanol with 1 mM lysozyme at 420 nm.

damage to the proteins. Since curcumin is known to be a very good scavenger of free radicals [20], we examined its ability towards the protein-derived free radicals. Pulse radiolysis technique was employed to study these reactions. Pulse radiolysis of N_2O-saturated aqueous solution containing 0.1 M sodium azide, 10% acetonitrite, 1 mM lysozyme, at pH 6.8 produced a species absorbing at 510 nm (Fig. 5). This species has been identified as the indolyl radical formed by the one-electron oxidation of the tryptophan in the lysozyme [11]. In the presence of curcumin, the lysozyme radical reacts with curcumin as seen by the increase in the formation of the transient at 510 nm with concomitant bleaching of curcumin at 430 nm (Fig. 6). The rate of formation of 510-nm absorption was monitored as a function of curcumin concentration from 23 to 125 μM and the bimolecular rate constant for the reaction of lysozyme radicals with curcumin was found to be 4.4×10^8 M^{-1} s^{-1}. Since lysozyme contains both tryptophan and tyrosine, this reaction of curcumin is probably due to the scavenging of these oxidized amino acids. Therefore, we have studied reactions of tryptophan and tyrosine radicals independently with curcumin. Fig. 7 shows the time-resolved spectrum of the transient obtained on pulse radiolysis of N_2O-saturated aqueous solution containing 1 mM tryptophan, 0.1 M N_3^- and 60 μM curcumin at pH 6.8. The solution contained 10% acetonitrile also to make the condition identical to that used when the concentration of lysozyme was low. It can be seen from Fig. 7 that indolyl radicals were formed in less than 2.5 μs after pulse irradiation. After 50 μs another transient formation at 510 nm was observed with concomitant bleaching at 430 nm. It appears that the tryptophan radical produced by one-electron oxidation by azide radical gets repaired in the presence of curcumin. The bimolecular rate constant for the repair of the indolyl radical with curcumin was found to be 5.0×10^8 M^{-1} s^{-1}. It therefore appears that curcumin reacts with oxidized tryptophan radicals with almost the same efficiency as it reacts with oxidized lysozyme. This could be due to the fact that tryptophan present

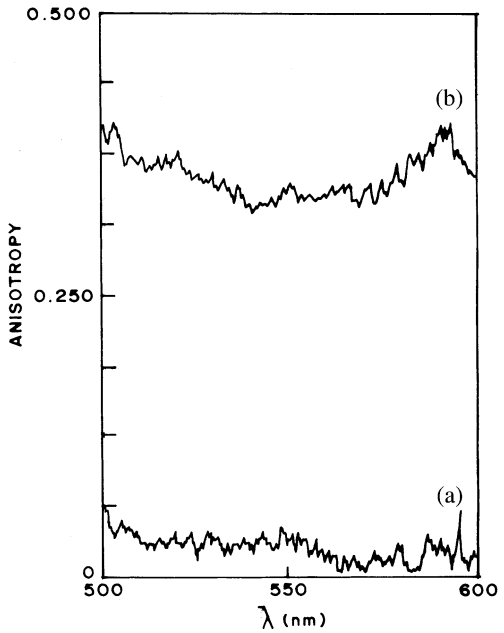

Fig. 4. Anisotropy measurements for solutions of 22 μM curcumin, $\lambda_{exc} = 420$ nm. (a) In aqueous solution containing 1% acetonitrile. (b) Same as (a) but in the presence of 1 mM lysozyme.

in lysozyme may be residing very close to curcumin binding site, hence is available readily for reaction with curcumin. However, no reaction was observed with tyrosine under identical experimental conditions.

Tryptophan has greater reactivity towards the azide radical than that of tyrosine [9]. The preferential oxidation of tryptophan in protein sets up the electron transfer to tyrosine from tryptophan radical. This is because the redox potential of tyrosine and tryptophan neutral species at pH 7 are reported as 0.94 and 1.05 V, respectively [28]. It has been suggested that the intramolecular electron transfer is much more predominant than intermolecular electron transfer in peptides [9].

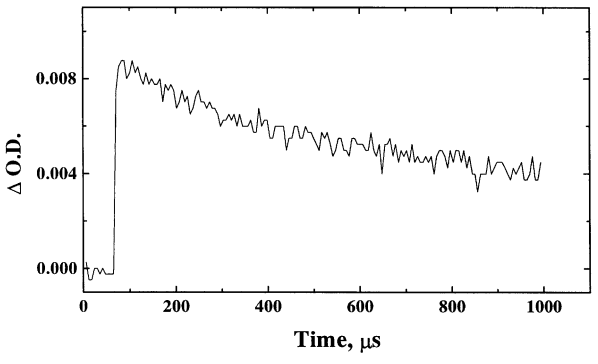

Fig. 5. Formation and decay of the indolyl radical in aqueous solution containing 1 mM Lz and 0.1 M NaN_3 at pH 6.8. The solution contained 10% acetonitrile.

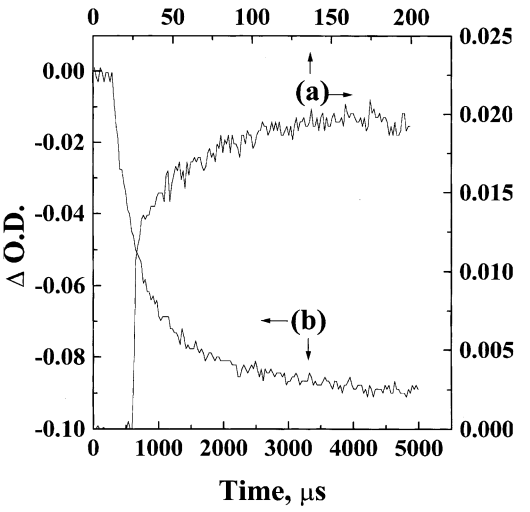

Fig. 6. Time-dependent (a) absorption growth at 510 nm and (b) bleaching of curcumin at 430 nm in N_2O-saturated aqueous solution containing 31 μM curcumin, 1 mM Lz and 0.1 M NaN_3 at pH 6.8. The solution contained 10% acetonitrile.

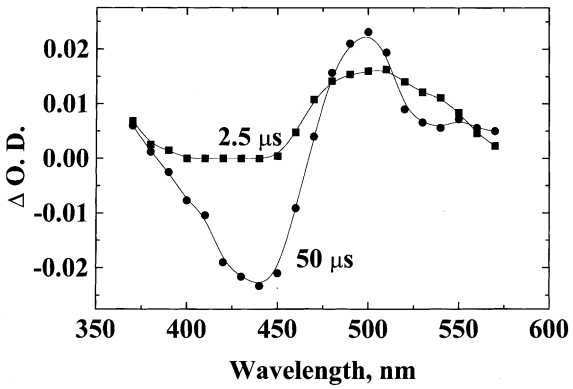

Fig. 7. Time-resolved transient absorption spectra obtained on pulse radiolysis of 1 mM tryptophan, 0.1 M NaN_3 and 60 μM curcumin at pH 6.8. The solution contained 10% acetonitrile.

The intramolecular electron transfer, which is independent of the concentration of peptides, is reported in the range of $2.7\times10^{8}-7.0\times10^{8}$ M^{-1} s^{-1}. The absence of any reactivity of tyrosine radical towards curcumin (see above) shows that the observed rate constant of curcumin reactivity towards lysozyme radical is mainly due to the reaction with the indolyl radical. Above results clearly show that curcumin can repair the indolyl radical. In addition, it can also inhibit intramolecular electron transfer in the proteins. The mechanism of repairing can be explained as below:

$$N_3^{\cdot} + \mathrm{TrypH} \rightarrow N_3^- + \mathrm{TrypH}^{+\cdot} \quad (6)$$

$$\mathrm{TrypH}^{+\cdot} \underset{pK_a=4.3}{\rightleftharpoons} \mathrm{Tryp}^{\cdot} + H^+ \quad (7)$$

Therefore, at pH 7, the tryptophan radical will be in the neutral form. In the presence of curcumin, neutral indolyl radical can react with it either by electron transfer or by H atom transfer. Very recently, it has been reported using empirical linear energy relations that the oxidation potential of curcumin at pH 7 is 0.77 V [29]. This is considerably lower than the redox potential of tryptophan and tyrosine. Hence, the electron transfer from indolyl radical to curcumin is thermodynamically favorable.

Thiols and disulfides are important biological compounds present in many proteins [11,30]. They are related to each other in a redox system with thiyl radical as the intermediate. The reaction of e_{aq}^- with bovine serum albumin results in a transient spectrum with a broad maximum at 420 nm and has been attributed to the addition of e_{aq}^- to disulfide groups. The disulfide radical anion ($RSSR^{-\cdot}$) so formed decays within 1 μs into the thiyl radical and thiolate:

$$\mathrm{RSSR} + e_{aq}^- \rightarrow \mathrm{RSSR}^- \rightarrow \mathrm{RS}^{\cdot} + \mathrm{RS}^- \quad (8)$$

Therefore, to see that whether curcumin protects the disulfide groups in protein we have carried studies with cystine as a model compound. Fig. 8 shows the formation of curcumin radical on reaction of disulfide radical anion with curcumin in a N_2-bubbled aqueous solution containing of 0.5 M *t*-butanol, 0.8 mM cystine and 0.5 mM curcumin at pH 6.8. Here it was easy to solubilize curcumin due to the presence of *t*-butanol. In this system e_{aq}^- reacts with disulfide to produce a $(RSSR)^{-\cdot}$ radical anion, which decays immediately, that is within 1 μs, into thiyl radicals and thiolate [30]. The formation of the curcumin radical is probably due to the reaction of curcumin with thiyl radicals via H atom transfer. It has been reported that curcumin acts as a better H atom transfer than thiols [29]. The bimolecular rate constant for the formation of curcumin phenoxyl radicals was found to be 3.7×10^{8} M^{-1} s^{-1} which is comparable to that observed earlier on reaction with glutathione radicals [31].

4. Conclusion

Curcumin, a lipid soluble phenol, has been showing many promising results both in vitro and in vivo systems. In this paper, we have shown for the first time the binding of curcumin to a protein i.e. curcumin exists in the close vicinity of the protein, by using spectroscopic methodology.

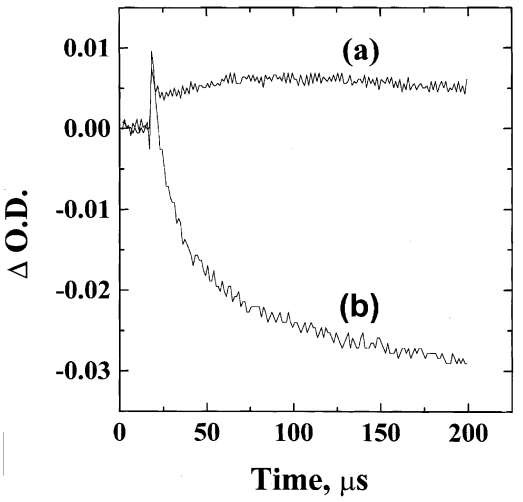

Fig. 8. Time-dependent (a) absorption growth of curcumin radical at 510 nm and (b) concomitant bleaching of curcumin at 430 nm in N_2-bubbled aqueous solution containing 75 μM curcumin, 0.8 mM cystine and 1.0 M *t*-butanol at pH 6.8.

However, the actual site of binding is difficult to predict based on these studies alone. As a result of such binding, curcumin is able to repair most of the oxidized amino acid, with a great efficiency. In addition, curcumin also reacts with thiol radicals. All these results indicate that curcumin can be a powerful antioxidant to repair both oxidative and reductive damage caused to proteins by radiation.

Acknowledgements

The authors are thankful to Dr J.P. Mittal, Director of Chemistry and Isotope Group and Dr T. Mukherjee, Head of Radiation and Chemical Dynamics Division, for their encouragement.

References

[1] O. Yamamoto, in: T.J. Ahren, M.C. Manning (Eds.), Chemical and Physical Pathways of Protein Degradation, Plenum Press, New York, 1992.
[2] J. Berges, E. Kassab, D. Conte, E. Adjadj, C. Houee-Levin, J. Phys. Chem. 101 (1997) 7809.
[3] W.M. Garrison, Chem. Rev. 87 (1987) 381.
[4] L.I. Grossweiner, Curr. Topics Radiat. Res. 11 (1976) 141.
[5] M.H. Klapper, M. Faraggi, Q. Rev. Biophys. 12 (1979) 465.
[6] W.A. Prutz, J. Butler, E.J. Land, A.J. Swallow, Biochem. Biophys. Res. Commun. 96 (1980) 408.
[7] W.A. Prutz, E.J. Land, Int. J. Radiat. Biol. 36 (1979) 513.
[8] W.A. Prutz, E.J. Land, R.W. Sloper, J. Chem. Soc. Faraday Trans. 1 (77) (1981) 281.
[9] M. Faraggi., M.R. DeFelippis, M.H. Klapper, J. Am. Chem. Soc. 111 (1989) 5141.
[10] S.V. Jovanovic, A. Harriman, M.G. Simic, J. Phys. Chem. 90 (1986) 1935.
[11] B.M. Hoey, J. Butler, Biochim. Biophys. Acta 791 (1984) 212.
[12] G.R. Buettner, Arch. Biochem. Biophys. 300 (1993) 535.
[13] J. Franke, F. Vogtle, Topics Curr. Chem. 132 (1986) 135.
[14] M.C.R. Symons, Radiat. Phys. Chem. 45 (1995) 837.
[15] N. Venkatesan, Br. J. Pharmacol. 124 (1998) 425.
[16] S.V. Singh, X. Hu, S.K. Srivastava et al., Carcinogenesis 26 (1998) 715.
[17] (a) V. Rajakrishnan, P. Viswanathan, K.N. Rajasekaran, G. Gunashekeran, V.P. Menon, Med. Sci. Res. 26 (1998) 715; (b) R.J. Anto, G. Kuttan, K.V.D. Babu, K.N. Rajasekaran, Int. J. Pharmacol. 131 (1996) 1.
[18] A.A. Gorman, I. Hamblett, V.S. Srinavasan, P.D. Wood, Photochem. Photobiol. 59 (1994) 389.
[19] C.F. Chignell, P. Bielski, K.J. Reszka, A.G. Motlen, R.H. Sik, T.A. Dahl, Photochem. Photobiol. 59 (1994) 295.
[20] K.I. Priyadarsini, Free Radical Biol. Med. 23 (1997) 838.
[21] N. Sreejayan, M.N.A. Rao, Int. J. Pharmacol. 100 (1993) 93.
[22] N. Sreejayan, M.N.A. Rao, J. Pharm. Pharmacol. 49 (1997) 105.
[23] M.K. Unnikrishnan, M.N.A. Rao, Mol. Cell Biochem. 146 (1999) 35.
[24] T. Mukherjee, in: S.A. Ahmad (Ed.), Atomic, Molecular and Cluster Physics, Narosa, New Delhi, 1997.
[25] P. Wardman, J. Phys. Chem. Ref. Data 18 (1989) 1637.
[26] S.M. Khopde, K.I. Priyadarsini, D.K. Palit, T. Mukherjee, Photochem. Photobiol. 72 (2000) 625.
[27] J. Franke, F. Vogtle, Topics Curr. Chem. 132 (1986) 135.
[28] M.R. DeFelippis, C.P. Murthy, M. Faraggi, M.H. Klapper, Biochemistry 28 (1989) 4847.
[29] S.V. Jovanovic, S. Steenken, C.W. Boone, M.G. Simic, J. Am. Chem. Soc. 121 (1999) 9677.
[30] K.D. Asmus, Methods Enzymol. B 186 (1990) 168.
[31] S.M. Khopde, K.I. Priyadarsini, P. Venkatesan, M.N.A. Rao, Biophys. Chem. 80 (1999) 85–91.