Role of lysine side chains in metallothionein

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Summary. pK_a-Values of lysine residues of mammalian metallothionein were determined by chemical titration measurements of ε-CH_2 lysine resonances in the ^{1}H-NMR spectra. They are about 0.5 pH-unit higher than the average pK_a-value of a metal-free derivative, suggesting interaction of the positively charged residues with the two three-fold negatively charged metal-thiolate clusters of the metal-containing form. Deprotonation of the lysines leads to circular dichroism changes attributable to an electrostatically induced structural transition of the protein.
Key words. Metallothionein; ^{1}H-NMR, circular dichroism; electrostatic interactions; lysine residues; pH-titration.

Introduction

Metallothionein is an ubiquitously occurring, extremely sulfur- and metal-rich protein (mol. wt 6000–7000) which plays a role in the metabolism and the detoxification of several essential and nonessential trace metals[19, 21, 29, 38]. This protein was first isolated from equine renal cortex by Margoshes and Vallee[23] in their search for a tissue constituent responsible for the accumulation of cadmium in animals. Subsequently, proteins with similar characteristics were also obtained from kidney, liver and intestines of a wide variety of mammals and recently also from birds, fishes, invertebrate organisms, plants[28], and from some eukaryotic microorganisms[21, 25]. An interesting feature of the metallothioneins is that their biosynthesis can be induced both by exposure to metals such as zinc, cadmium, mercury, and others[25, 30] and by the administration of glucocorticoid hormones[14]. All mammalian forms characterized to date contain a single polypeptide chain with a total of 61 amino acid residues out of which 20 are cysteines[22]. There are seven g-atoms of Zn and/or Cd bound per chain weight of 6100. Another unusual feature of metallothionein is the lack of aromatic amino acids[22]. Spectroscopic measurements[18] and studies by x-ray photoelectron spectroscopy[39] have shown that all 20 cysteine residues participate in metal binding via mercaptide linkages and that there are no disulphide bridges in the molecule. Recent ^{113}Cd NMR measurements on ^{113}Cd metallothionein[26] and electron spin resonance and magnetic susceptibility measurements on Co(II)-metallothionein[32] have clearly established the presence of thiolate cluster structures in this protein.

We have demonstrated that a wide variety of metal ions can be reproducibly incorporated into metallothionein[31, 32, 34, 36]. It is conceivable that amino acids other than those which act as ligands to the metal may be important in stabilizing the structure of the negatively charged metal-thiolate complexes. Based on sequence data, the involvement of basic amino acids in the stabilization of the metal-binding sites via outer-sphere complexes has been suggested[22]. The present paper describes ^{1}H-NMR and CD[4] studies which provide some evidence that the lysines are involved in this way.

Materials and methods

Rabbit liver metallothionein-2 was isolated from rabbits injected with cadmium chloride (by 20 s.c. injections of 1 mg Cd/kg b.wt at intervals of 2–3 days)[20] and was purified by a modification of the procedure of Bühler and Kägi[8]. Equine kidney metallothionein-1A was isolated by the method of Kägi et al.[19]. The puritiy of each preparation was checked by determining the amino acid composition (Durrum D-500 analyzer)[20] and by metal analysis using atomic absorption spectroscopy (64% Cd, 36% Zn for rabbit metallothionein-2 and 59% Cd, 41% Zn for equine kidney metallothionein-1A) (Instrumentation Laboratory model IL 157). Protein concentration was determined spectrophotometrically by measuring the absorption of thionein at 220 nm in 0.1 M HCl using an absorption coefficient $A_{220} = 7.9$ mg cm^{-1} ml^{-1}[19]. All chemicals were of reagent grade or better. Buffer solutions were extracted with dithizone in CCl_4 or passed over a Chelex-100 (Bio-Rad Corporation) to remove trace metal contaminants. Absorption spectra were recorded on Perkin-Elmer model 340 spectrometers using 1.0 cm pathlength quartz cells. Molar absorptivities, ε, are based on the protein concentration and are expressed in units of M^{-1} cm^{-1}. CD spectra were recorded at room temperature (23 °C) on a Cary 61 spectropolarimeter using 0.02 and 0.1 cm pathlength quartz cells. All CD measurements were performed using the 0.05 M Trizma base buffer, the required pH was adjusted either by 0.1 M HCl or by 0.1 NaOH. CD is expressed in terms of molecular ellipticity $[\theta]$ and is referred to the protein concentration with units of deg cm^2 $dmol^{-1}$. ^{1}H-NMR spectra were recorded at 300 K on a Bruker 300 MHz FT spectrometer. In the spectra used for pH titration the convolution difference technique was used to improve spectral resolution[11]. Protein solutions for the NMR experiment were rendered free of Trizma base by dialysis against three changes of 1 mM sodium phosphate buffer, pH 7.5, followed by lyophilization. The dialysis tube used had a molecular weight cut-off of 3500 (Spectrapor Co). The lyophilized solid was dissolved in 20 mM sodium phosphate in D_2O, pH 7.5. The protein solutions were allowed to equilibrate with D_2O at room temperature for 4 h in order to facilitate deuteration of all exchangeable protons.

A Pye Ingold microelectrode attached to a Radiometer pHmeter 26 was used for pH measurements. NaOD solutions were used for pH adjustments. Since to a good approximation the pH values for peptides and biomolecules in D_2O correspond to those in H_2O[10], the values of direct pH-meter reading were used and quoted as pH. The residual water peak in the NMR experiment

was suppressed by selective irradiation before application of the measurement pulse. All chemical shifts are reported as values downfield of the internal standard DSS[4].

Results

Since aromatic amino acids and histidine are absent in mammalian metallothioneins[18, 22], only the high-field regions (0–5 ppm) of the ^{1}H-NMR spectra are shown. Figure 1 shows the ^{1}H-NMR spectrum of rabbit metallothionein-2 (fig. 1b) and a random coil spectrum using an amino acid mixture having the same composition as rabbit metallothionein-2 (fig. 1a). The two spectra are comparable, indicating that the majority of the individual resonances are unperturbed by protein folding[37]. When the pH of solutions of either rabbit or equine metallothioneins is raised from 7.5 to 11.2, changes occur at about 3.0 and 1.7 ppm (fig. 1c, peaks A and B), implying perturbation of the ε- and of the overlapping β- and δ-CH_2 resonances, respectively, of lysine residues which are located in these regions[12, 40]. The peaks at 3.0 and 1.7 ppm are shifted upfield as expected on the loss of positive charge by deprotonation – in this case from the lysine NH_3^+. Because of the potential overlapping with the cysteine β-CH_2 resonances in the spectrum of the amino acid mixture (fig. 1a), the identification of the signals due to the lysine ε-CH_2 protons may seem ambiguous. However, as revealed by a recent 2D ^{1}H-NMR study, the cysteine β-CH_2 resonances of metallothionein have an extremely wide spread (> 1 ppm) and, hence, they are not causing any major overlap with the 3.0 ppm signal of lysine[24]. This is also in agreement with quantitative measurements. Thus, integration of the pH-shifted 3.0 ppm signal gives a total of 16 ± 2 protons in rabbit metallothionein-2 consistent with the eight lysine residues present in the protein. Further support for the assignment of the pH-shifted 3.0 ppm resonances to lysine protons comes from decoupling experiments. Saturation of the resonances in the 3.0 ppm region resulted in changes in the broad resonance at about 1.7 ppm assigned to the overlapping β- and δ-lysine CH_2 protons.

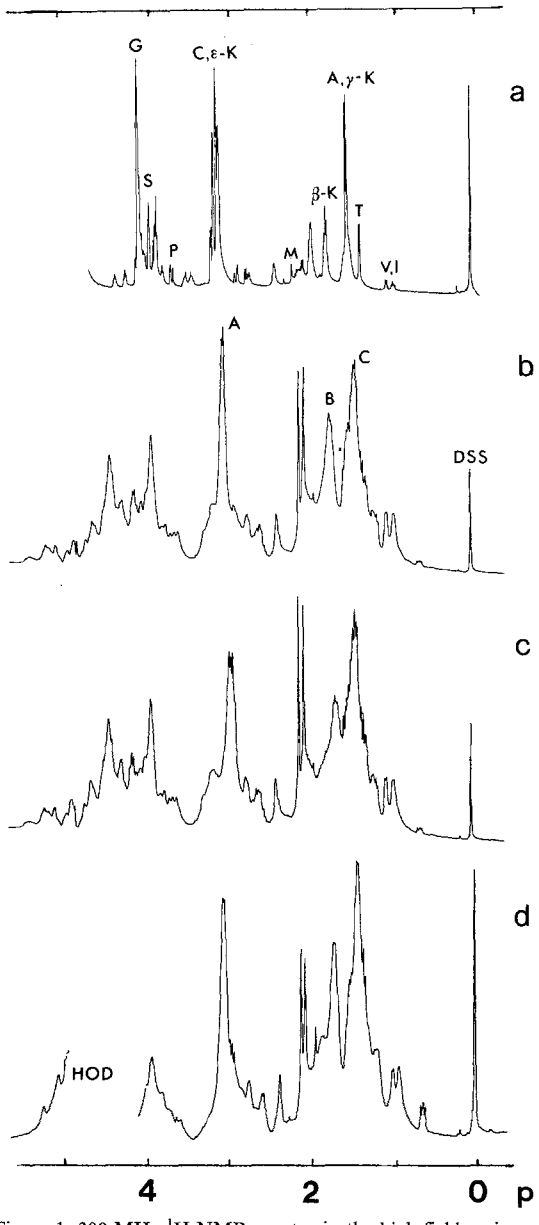

Figure 1. 300-MHz ^{1}H-NMR spectra in the high-field region of 2 mM rabbit (Cd, Zn)-metallothionein-2 in ^2H_2O containing 20 mM sodium phosphate buffer. *a* Amino acid mixture having the same composition as rabbit metallothionein-2, pH 7.9. *b* Metallothionein at pH 7.9. *c* Metallothionein at pH 11.0. *d* Metallothionein in 4 M sodium perchlorate at pH 7.9. The labeled peaks are: DSS (2,2-dimethyl-2-silapentane-5-sulphonate) internal standard; HOD, residual water peak; A, lysine ε-CH_2 resonances; B, lysine β- and δ-CH_2 resonances; C, lysine γ-CH_2 resonances. The remaining labels in the spectrum of amino acid mixture refer to main amino acid residues contributing to the resonances marked in common one-letter code. The signal marked β-K includes both the β- and δ-CH_2 resonances of lysine.

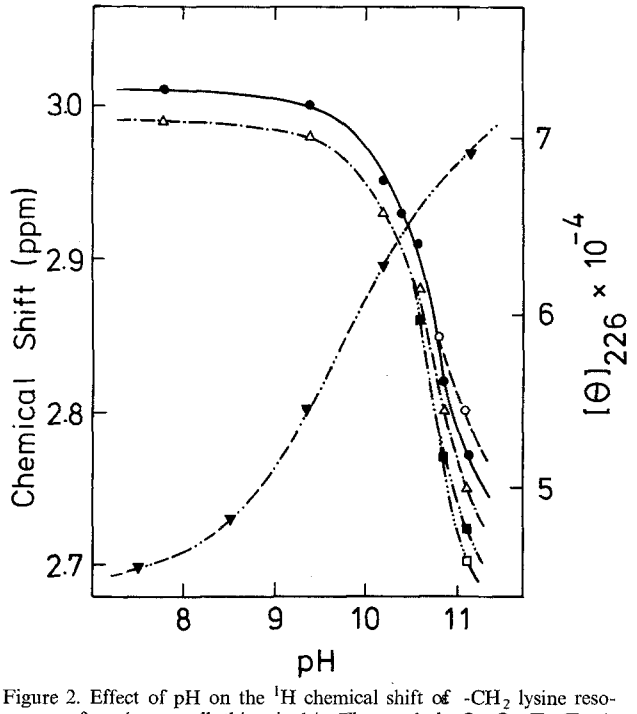

Figure 2. Effect of pH on the ^{1}H chemical shift of α-CH_2 lysine resonances of equine metallothionein-1A. The symbols ●, ○, ■, □, △ refer to different resolved resonances. Also plotted is the pH-dependent change in molecular ellipticity at 226 nm (▼).

As mentioned above, the high-field shift of the lysine resonances is due to deprotonation of the lysine ammonium group. A plot of the position of the ε-CH_2 signals against pH (fig. 2) indicates that the major change occurs between pH 10 and 11, consistent with the range of pK-values of lysine residues in proteins[13]. Another significant feature is that the spread of the chemical shifts of the ε-CH_2 lysine resonances increases as the pH is raised (fig. 1c). While at neutral pH only two peaks are resolved, at pH 11 a total of five unresolved triplets are observed for equine metallothionein-1A, extending over a range of 0.1 ppm. This spread is most likely due to the various lysine residues having slightly different pK_a's. Plots of the various chemical shifts in function of pH are given in figure 2, yielding a family of tentative titration curves. However, due to the occurrence of irreversible changes in the structure above pH 11.5 (indicated by changes in the absorption spectrum), the deprotonation of the primary ammonium group of the lysine residues could not be followed to completion. Since metallothionein does not possess any other titratable group(s) above pH 5, the NMR titration curves both of interacting and of noninteracting lysine side chains should be symmetrical[5] though. Accordingly, the individual pK_a's were derived from the first derivatives of the partial titration curves resulting in values ranging from 10.6 to 10.9.

Because of the lack of aromatic amino acids and, hence, of ring-current effects, the high-field region of the ^{1}H-NMR spectrum of metallothionein is relatively insensitive to conformational transitions[37] and, hence, any structural changes accompanying deprotonation of the lysine residues are difficult to detect by the ^{1}H-NMR method employed in this study. However, evidence for the occurence of conformational changes in metallothionein on deprotonation of the lysine residues comes from CD measurements. The CD spectrum of native rabbit (Cd, Zn)-metallothionein-2 at different pH's is shown in figure 3a. The spectra are dominated by contributions from the metal-thiolate clusters which are also prominent in the absorption spectra of metallothioneins (fig. 3c)[34]. The absorption spectra of (Cd, Zn)-metallothionein include at least six metal-thiolate transitions (fig. 3c) of which at least three are overlapping with the intrinsic absorption bands of the polypeptide chain. The same overlapping occurs in some of the CD bands (fig. 3a, 3b). Thus, the extrema at 203 nm ($[\theta] = -688{,}000$) and at 226 nm ($[\theta] = +48{,}000$) are the resultant of ellipticity contributions from protein and from metal-thiolate chromophores. The extrema at 239 nm ($[\theta] = -91{,}000$) and 259 nm ($[\theta] = +159{,}000$) arise solely from the metal-thiolate chromophores [36].

On going from pH 7.5 to 11.2 the bands at 203 nm and 226 nm become more intense while the two low energy bands remain unaltered. Since the absorption spectrum is also unchanged, it is a reasonable inference that the CD changes reflect alterations in polypeptide conformation only. The pH-dependent changes in the CD spectrum are fully reversible. A plot of the ellipticity at 226 nm vs pH gives a titration curve with a pK-value close to those of the lysine side chains (fig. 2). The direction of the ellipticity change at 226 nm is the same as that observed on addition of high concentrations of 6 M guanidine·HCl[15, 33] and may thus reflect a loss of some hydrogen-bonded secondary or tertiary structure upon deprotonation of the lysine residues[6, 16, 17].

The structure of metallothionein is also markedly affected by changes in ionic strength as indicated by the loss in ellipticity at the 203 nm and 226 nm extrema on going from 0.1 to 3.0 M $NaClO_4$ or KCl (fig. 3b). There are also discrete changes in the ^{1}H-NMR spectrum. An increase in salt concentration from 0.1 M to 4.0 M $NaClO_4$ or KCl results in a marked sharpening of the 3.0, 1.7 and 1.5 ppm signals of rabbit metallothionein-2 (fig. 1d, peaks A, B and C). It suggests that at high ionic strength some of these residues are becoming more freely exposed to the solvent.

Discussion

From the high degree of conservation of the basic amino acid residues in all mammalian metallothioneins

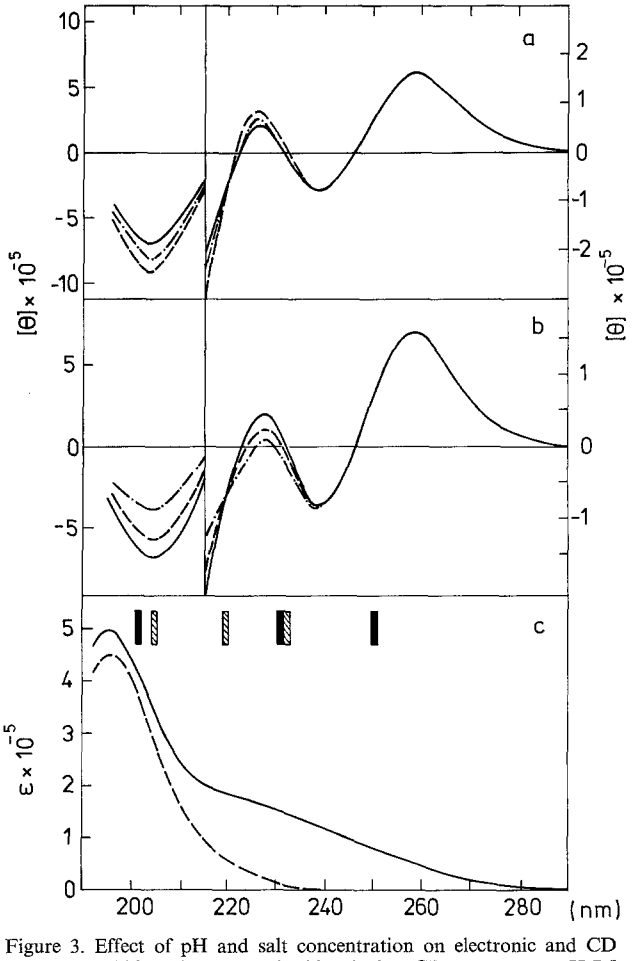

Figure 3. Effect of pH and salt concentration on electronic and CD spectra of rabbit (Cd, Zn)-metallothionein-2. *a* CD spectrum at pH 7.5 (full line), at pH 9.3 (stippled line) and at pH 11.2 (dashed line). For the sake of clarity, the spectra obtained at pH 8.6 and 10.3 were omitted. *b* CD spectrum (in 0.05 M Tris·HCl buffer, pH 8.0) without added salt (full line), with 1 M $NaClO_4$ (dashed line), and with 3 M $NaClO_4$ (stippled line). Identical CD profiles were obtained when KCl was used instead of $NaClO_4$. *c* Absorption spectrum of thionein (apoprotein) at pH 1 (dashed line) and of metallothionein at pH 7.5 (full line). The shaded and full bars indicate the positions of the resolved metal-induced transitions of zinc-metallothionein and cadmium-metallothionein, respectively[34].

it has been suggested previously[22] that these residues play an important role in charge neutralization at the metal-thiolate centers. In the now accepted model of two separate metal-thiolate clusters built of tetrahedral tetrathiolate units[26,31,32], the cysteine-to-metal stoichiometry generates a total of six negative charges which must be neutralized by appropriate counter ions. The juxtaposition of all basic amino acid residues to the metal-chelating cysteine sequences predisposes them for balancing the charges by forming outer-sphere complexes. Such an electrostatic interaction would be expected to stabilize the cluster and to manifest itself by a shift of the pK_a's of the lysine amino groups towards higher values. That this is indeed the case is indicated by the ^{1}H-NMR titration profiles of the ε-CH_2 resonances which have their midpoints between pH 10.6 and 10.9, i.e., in a range above that expected for freely exposed lysine side chains[13] (fig. 2). That the formation of metal clusters shifts the average pK_a of the lysine residues from the reference value of 10.3 measured in the metal-free S-carboamido derivative of the protein to 10.9 in metallothionein was also shown independently by potentiometric measurements[15]. In line with their abnormal titration behavior, the lysine residues of metallothionein display a lowered chemical reactivity. Thus, all eight lysines of the metal-containing protein react more slowly with TNBS[4] than those of the metal-free S-carboamido derivative [27].

The importance of electrostatic contributions to the structure of metallothionein is also emphasized by the effects of changes in ionic strength on the magnetic resonance and optical activity properties. The sharpening in the region of the lysine ε-CH_2 and of the lysine β- and δ-CH_2 resonances brought about by raising salt concentrations (fig. 1d) can be attributed to an increase in the degree of exposure of the lysine side chains to the solvent, thereby rendering them magnetically more equivalent. The effect of increasing ionic strength on ellipticity closely parallels the changes observed on protonation of the lysine amino group (fig. 3). At high pH, a high salt concentration can compensate in part the electrostatic repulsion brought about by the loss of the salt bridges with lysine. At neutral pH, the salt effect may reflect a reinforcement of the local charge compensation. That such electrostatic interactions are of fundamental significance for the maintenance of the gross structure of metallothionein is also evident from the recent finding that a change in ionic strength from 0.01 to 0.5 causes as 20% reduction in Stokes' radius indicating a massive change in molecular shape or hydration[35]. Thus, the present data allow the inference that the positive charges of the lysine residues play an important role in restraining this structural expansion of the molecule.

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4 Abbreviations used: CD, circular dichroism; DSS, 2,2-dimethyl-2-silapentane-5-sulphonate; TNBS, 2,4,6-trinitrobenzene sulfonic acid.
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Short Communications

A new sterol from the sponge *Haliclona chilensis* (Thiele)

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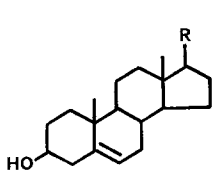
Summary. From the sponge *Haliclona chilensis* (Thiele) a minor keto-sterol was isolated and characterized as 24-keto-cholesta-5,25-dien-3β-ol by means of spectroscopic (^{1}H and ^{13}C-FT NMR, MS) methods.
Key words. Sponge; *Haliclona chilensis;* 24-keto-cholesta-5,25-dien-3β-ol; sterol.

Research on sterol components of marine invertebrates has shown that several species of lower organisms contain a variety of sterols with modified side chains[1,2]. Continuing with our investigations on sterols from aquatic organisms[3] we studied the sterol fraction of the sponge *Haliclona chilensis* (Thiele) which was collected at Chiloé, Chile, in March 1983 and frozen immediately. Work up was done as previously described[3]. As well as sterols that are already known[4], separated by chromatography on silver-nitrate-impregnated silica gel, the fraction contained a new sterol **1** which was purified as acetate by reverse phase HPLC (Whatman Partisil ODS-2) with RRT 0.35 (cholesterol acetate = 1 in abs. methanol) and was homogeneous by GC analysis (12 m × 0.02 mm fused silica capillary column coated with methylsilicone, Hewlett-Packard, 200–280°, 8/min) with RRT 1.19 (cholesterol acetate = 1). Its mass spectrum (398, M^+) displayed the typical pattern of Δ^5-3β-hydroxy sterols[5] (m/z 213, 231 and 253) and the base peak at m/z 271 suggested the presence of unsaturation in the side chain. Its IR spectrum presented a band of α,β-unsaturated ketone at 1680 cm^{-1} that should be located at the side chain. The ^{1}H-FT NMR spectrum (table) showed the presence of a terminal vinyl



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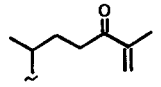

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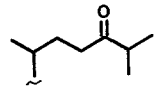

^{1}H-FT NMR (100 MHz, $CDCl_3$)		^{13}C-FT NMR (25.2 MHz, $CDCl_3$, TMS as int.st.)					
H	δ ppm (TMS)	C	δ ppm	C	δ ppm	C	δ ppm
3	4.62 (m)	1	36.95t	11	21.01t	21	18.54q
6	5.38 (m)	2	27.74t	12	39.67t	22	30.74t
18	0.68 (s)	3	73.85d	13	42.30s	23	34.36t
19	1.02 (s)	4	38.08t	14	56.59d	24	202.00s
21	0.94 (d, J = 6 Hz)	5	139.45s	15	24.23t	25	144.39s
26a*	5.76 (m)	6	122.41d	16	28.10t	26	123.95t
26b*	5.96 (m)	7	31.83t	17	55.81d	27	17.70q
27	1.87 (b.s.)	8	31.83d	18	11.85q	CH_3CO	21.37q
CH_3CO	2.04 (s)	9	49.95d	19	19.28q		
		10	36.54s	20	35.45d		

* Determined by double irradiation.