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INTERACTION OF CYSTEAMINE WITH THE THIOL AND DISULPHIDE GROUPS IN DEOXYRIBONUCLEOPROTEINS

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Abstract—The thiol content of deoxyribonucleoproteins (DNP), isolated from Ehrlich ascites tumor cells by 1 M NaCl extraction, was measured by amperometric titration with $AgNO_3$ before and after reduction of the disulphide bonds with $NaBH_4$. The amount of –SH and SS groups was 59 μmoles and 19 μmoles/g protein, respectively. About 20 μmoles –SH/g protein of the total thiol content of DNP could be extracted with a weak acid after reduction of the disulphide bonds. In addition to proteins, the acid soluble fraction contained also glutathione and unidentified –SH containing polypeptides with molecular weights larger than glutathione. The radioprotective compound cysteamine was found to form mixed disulphides with the –SH groups of DNP, and to reduce existing disulphide bonds and thereby release DNP-bound peptides. The possible importance of –SH and disulphide groups in DNP for radiation protection is discussed.

FOR MANY years it has been generally accepted that deoxyribonucleoproteins (DNP) are more or less devoid of cysteine.[1] In many investigations only trace amounts of cysteine could be found after acid hydrolysis and subsequent amino acid analysis. During recent years, however, several studies clearly demonstrated the presence of thiol and disulphide groups in histones, notably in the arginine-rich F3 fraction. Philips[2] found that the F3 fraction of calf thymus histones has an –SH content of 60 μmoles/g protein, and the fractions F1, F2a and F2b have amounts varying between 3 and 24 μmoles –SH/g protein. Similar results were obtained by Jellum,[3] while Hilton and Stocken[4] found an –SH content in the range 38–51 μmoles/g protein in the F3 fraction. It would now seem that cells derived from vertebrates up to and including rodents contain a single cysteine residue in their F3 histone, while the cells from more highly evolved mammals contain two cysteine residues per F3 histone molecule.[5]

The sulphydryl groups of histones may play an important role in the control of DNA function concerning such processes as RNA synthesis and cell replication.[4,6] Evidence is accumulating which suggests that the interaction of radioprotective sulphydryl containing compounds with nuclear constituents, such as DNA and nucleoproteins may be of importance for the radioprotective mechanism.[7,8] Furthermore, cysteamine treatment has been found to induce repair in cells irradiated under anoxic conditions, when in the absence of cysteamine repair does not take place.[9]

The present study was undertaken with the purpose to identify and to obtain a quantitative measure of the content of sulphydryl, disulphide and mixed disulphide groups in the nucleoproteins of a mammalian cell material, and to examine how and to what extent the radioprotective compound cysteamine interacts with these sulphydryl groups.

MATERIALS AND METHODS

Cells. A hyperdiploid Ehrlich ascites tumor, denoted ELD, was used. It was carried by weekly intraperitoneal passages in male, 8–10-week-old, hybride mice. Tumors solely with a minimum concentration of 160 × 10^6 cells/ml ascites fluid and without blood contamination were used. Cell counts were routinely made with an electronic particle counter (Coulter Counter Model B).

Chemicals. ^{35}S-cysteine (sp. act. 30 mCi/mmole) and ^{35}S-cysteamine (sp. act. 4 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, England. Sephadex-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of highest purity commercially available. Solutions were made, in all cases, with demineralized water in order to reduce possible auto-oxidation of –SH groups by trace metals.

Isolation of nuclei. Cells from freshly withdrawn ascites tumors, grown for 5–7 days, were pooled and washed twice in 20 vol. of a balanced salt solution (BSS) by gentle centrifugation. The cell membrane was disrupted by osmotic shock in diluting the cell suspension 1:6 with distilled water followed by gentle shaking for 10 min. After centrifugation the disrupted cells were resuspended in a 0·25 M sucrose medium containing 0·05 M EDTA, layered on top of 0·88 M sucrose and centrifuged for 15 min at 1200 *g*. The nuclei were collected at the bottom of the centrifuge tube. The residue found at the interphase of the 0·25 and 0·88 M sucrose was resuspended in 0·25 M sucrose, and subjected to another centrifugation. The collected nuclei were resuspended in BSS. Microscopic examination revealed that this procedure yields a homogenous suspension of pure nuclei.

Isolation of deoxyribonucleoprotein. The method described by Wang[10] was followed. The isolated nuclei were homogenized in a glass homogenizer with a teflon pestle in buffered saline (0·14 M NaCl–0·005 M $MgCl_2$–0·02 M Tris buffer, pH 7·4) and centrifuged in a Spinco ultracentrifuge at 20,000 *g* for 15 min. The pellet was once more extracted with buffered saline and centrifuged. It was then homogenized with 1 M NaCl and the resulting suspension gently agitated in the cold (4°) for 12 hr and then centrifuged at 90,000 *g* for 1 hr. The clear supernatant was diluted to a salt concentration of 0·14 M NaCl with 6 vol. of demineralized water in order to precipitate the DNA–protein complex (DNP). After renewed centrifugation at 20,000 *g* for 15 min, this complex was dissolved in 0·125 M Tris buffer, pH 7·4, containing 8 M urea, for use in subsequent determinations of thiol and disulphide groups.

Determination of –SH groups and disulphide bonds. Non-protein bound –SH groups (NPSH) were determined with the DTNB reagent (5,5′-dithiobis-2-nitrobenzoic acid) according to Ellman.[11] Protein bound –SH groups (PSH) were assayed by an automatic amperometric titration procedure.[12] Reduction of disulphide bonds with sodiumborohydride ($NaBH_4$), a compound which exclusively splits disulphide bonds, and separation of protein and low-molecular acid-soluble thiols, were performed as described in an earlier paper.[12]

Cysteamine treatment. Freshly withdrawn and washed tumor cells were incubated at a concentration of 80–100 × 10^6 cells/ml in a tissue–culture medium containing 10 mM unlabelled or ^{35}S-labelled cysteamine (sp. act. 6–8 × 10^8 counts/min/mmole) at 37° for 30 min. After treatment, the cells were washed twice with fresh medium by gentle centrifugation, and the nuclei and subsequently the nucleoproteins were isolated as described above.

Chromatographic analysis. The low-molecular thiol-containing extract, obtained after reduction of the disulphide bonds, was subjected to gel-filtration. The sulphur of the nucleo–proteins had in this case been isotope-labelled by intraperitoneal injection of 50 μCi of ^{35}S-cysteine into the tumor-bearing animals 24 hr before harvesting the tumor cells. Five ml portions of the solution were passed through a Sephadex-25 (fine grade) column (2·5 × 45 cm), equilibrated with 0·125 M Tris buffer, pH 7·4. The flow-rate was 30 ml/hr, and the eluate was collected in 3 ml fractions. The procedure was carried out at 4°.

Radioactivity assay. ^{35}S-radioactivity was assayed by liquid scintillation spectroscopy in a Packard Tri-Carb scintillation counter Model 500-B. Aliquots to be assayed were added in 0·5 ml portions to a scintillation fluid containing naphthalene, PPO and POPOP in an ethanol, dioxane, toluene solvent system. The scintillation fluid could take 0·6 ml of aqueous solution per 15 ml without phase separation. Quenching factors were calculated by the addition to the samples of a standard amount of ^{35}S-cysteine. Protein containing samples were dissolved in formic acid before radioactivity assay.

Protein and DNA determination. Protein concentration was estimated according to the method of Lowry *et al.*[13] and DNA according to Burton.[14]

RESULTS

The total amount of –SH groups was determined before and after $NaBH_4$ reduction of the disulphide-bonds in deoxyribonucleoproteins (DNP) prepared from Ehrlich ascites tumor cells. As an indicator of the purity of the DNP preparations, the ratio of the amount of protein to DNA was calculated. These ratios, the effect of disulphide reduction on the content of –SH groups in DNP, and the amount of thiols extractable from reduced DNP by a weak acid (6% perchloric acid), are shown in Table 1.

TABLE 1. THE PROTEIN TO DNA RATIO AND THE CONTENT OF –SH GROUPS IN DNP FROM UNTREATED EHRLICH ASCITES TUMOR CELLS OR AFTER INCUBATION OF THE CELLS WITH CYSTEAMINE, NEM OR NEM FOLLOWED BY CYSTEAMINE*

Treatment	No. repeated exp.	Ratio Protein/DNA	Sulphydryl groups in DNP (μmole –SH/gprotein)		
			Before reduction	After reduction	
				Total	Acid soluble fraction†
None	6	1·30 ± 0·04	59·5 ± 3·2	97·5 ± 6·2	20·7 ± 2·2
10 mM Cysteamine	7	1·16 ± 0·05	39·8 ± 1·8	97·2 ± 4·6	33·2 ± 1·2
10 mM NEM	4	1·26 ± 0·04	15·2 ± 1·1	57·6 ± 4·5	19·4 ± 2·1
10 mM NEM + 10 mM cysteamine	4	1·21 ± 0·05	12·5 ± 1·2	65·3 ± 5·3	22·4 ± 1·8

* Analysis was performed before and after reduction of the disulphide bonds with $NaBH_4$ in the presence of 8 M urea.

Mean ± S.E. is indicated.

† The reduced DNP solution was extracted with 6% perchloric acid.

Before reduction of the disulphide bonds, the –SH content of DNP was 59·5 μmoles/g protein. After reduction it increased to about 97 μmoles/g. About 20 per cent

of this amount, i.e. 20·7 μmoles/g could be recovered in the supernatant after treatment of the protein sample with a weak acid.

In order to identify the thiols in the acid soluble fraction, the sulphur of DNP was isotope-labelled by pretreatment with ^{35}S-cysteine. Figure 1 illustrates the chromatographic pattern obtained by gel-filtration of the neutralized acidic extract, admixed

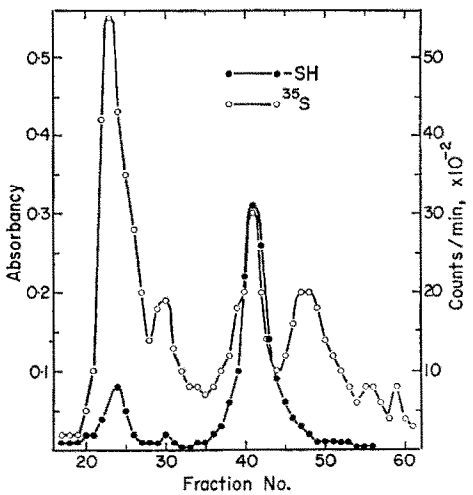

FIG. 1. Chromatographic pattern of the acid soluble fraction of ^{35}S-labelled DNP, the disulphide bonds of which were reduced by $NaBH_4$ treatment. Glutathione was added as reference substance. The concentrations of -SH groups and ^{35}S radioactivity in 3 ml fractions are illustrated.

with 0·5 μmoles glutathione (GSH) as reference substance. The concentration of -SH groups and ^{35}S-radioactivity was measured in 3 ml fractions. Four peaks can be distinguished in the pattern of the isotope-labelled material. As has been shown in previous studies,[12] the first peak with an elution point around fraction 23 corresponds to protein, and the fourth peak with an elution point around 48 to cysteine with a high specific ^{35}S-activity. The peak with an elution point around 30 probably represents large polypeptides which have earlier been demonstrated to be bound to DNP,[16,17] and the peak around point 41 corresponds to the reference substance, i.e. GSH. Since the incorporation of ^{35}S-cysteine into the different cellular constituents most likely proceeds at different rates, the peaks can only be used for identification purposes, and the radioactivity does not necessarily indicate quantitative proportions.

The binding of cysteamine to DNP was studied by incubating the tumor cells with 10 mM ^{35}S-labelled cysteamine for 30 min. This treatment resulted in a reduction in the amount of -SH groups in DNP to 39·8 μmoles/g protein, i.e. a decrease of about 33 per cent. After reduction of the disulphide bonds, the -SH content increased to about 97 μmoles/g protein, out of which 34 per cent, i.e. 33·3 μmoles/g could be extracted by treatment with a weak acid (Table 1). This amount of extractable thiols is larger in comparison to those extractable from the reduced DNP of untreated cells and is, obviously, attributable to the release of protein-bound cysteamine. From the specific activity of cysteamine (7×10^5 counts/min/mmole -SH) and the mean

TABLE 2. AMOUNT OF ^{35}S-RADIOACTIVITY BOUND TO DNP AFTER TREATMENT OF EHRLICH ASCITES TUMOR CELLS WITH ^{35}S-CYSTEAMINE OR NEM FOLLOWED BY ^{35}S-CYSTEAMINE*

Treatment	No. repeated exp.	^{35}S-activity in DNP		^{35}S-activity in acid soluble fraction
		Before reduction	After reduction	After reduction
		(counts/min × 10^{-4}/g protein)		
10 mM Cysteamine†	7	1260 ± 69 (18·0)	169 ± 34 (2·4)	953 ± 83 (13·6)
10 mM NEM + 10 mM cysteamine	4	530 ± 61 (7·6)	112 ± 32 (1·6)	364 ± 46 (5·2)

* Measurements were made before and after reduction of the disulphide bonds with $NaBH_4$ in the presence of 8 M urea. The acid soluble fraction was extracted with 6% perchloric acid. Mean ± S.E. is indicated.

† Specific activity 7 × 10^5 counts/min/mmole.

‡ Figures in parentheses indicate the amount of protein bound cysteamine per micromole/gram of protein.

radio-activity of DNP (1260 × 10^4 counts/min/g protein) it could be calculated that about 18 μmoles cysteamine was attached to DNP/gram (Table 2).

Table 2 also indicates that, upon reduction of the disulphide bonds, 80 per cent of the ^{35}S-radioactivity could, on the average, be recovered in the acid soluble fraction, i.e. was released from DNP. The remaining part appears to be firmly bound, suggesting that cysteamine can be attached to DNP also in some other way than by the formation of disulphide bonds.

In order to study whether disulphide formation between DNP and cysteamine is due to the binding of cysteamine to the –SH groups of DNP alone, or also to an interaction with the disulphide groups of DNP, the cells were pretreated with a thiol-blocking agent, *N*-ethylmaleimide (NEM) in 10 mM concentration before exposure to cysteamine. A comparison of the –SH content of DNP from untreated cells with that of NEM-treated cells shows that NEM treatment decreased the amount of –SH groups to 15·2 μmoles/g, i.e. resulted in a decrease by 75 per cent (Table 1). Treatment with NEM concentrations above 10 mM did not further decrease the –SH content, suggesting that some 25 per cent of the –SH groups were not available for binding with NEM due, probably, to the tertiary structure of the proteins. These hidden –SH groups became, however, available for determination upon protein denaturation which routinely precedes the –SH measurements. A similar finding was obtained by Jellum[3] who suggested that the –SH groups which remain free after NEM-treatment, actually are attributable to an unspecific binding of Ag ions to the protein during the amperometric titration of PSH. However, this may not be true, since amperometric titration with $AgNO_3$ of proteins with known content of –SH groups gives correct values.[12]

Upon disulphide reduction, the –SH content of NEM-treated DNP increased to 57·6 μmoles/g protein. This increase is similar to the increase of the –SH content when DNP from untreated cells is reduced. After treatment with a weak acid, again an amount of 20 μmoles –SH/gram of protein could be recovered in the supernatant as was the case with the NEM-untreated samples.

Cysteamine exposure following NEM treatment resulted in only a slight further

decrease of the –SH content of the unreduced DNP. Upon disulphide reduction, the increase of the –SH content was only insignificantly larger than with NEM treatment alone. A slight increase was also noted in the thiol content recoverable by treatment of the reduced DNP with weak acid (22·4 μmoles/g). It can be calculated from the specific activity of cysteamine and the activity of DNP (Table 2) that 7·6 μmoles cysteamine/g of protein was attached to DNP after NEM treatment. Upon reduction of the disulphide bonds, about 75 per cent of the activity was released and recovered in the acid soluble fraction.

DISCUSSION

The ratio protein–DNA in the DNP preparations used in this investigation was in the range 1·16–1·30. In nuclei isolated from tissues in which rapidly dividing cells predominate, a histone–DNA ratio around 1·0 has been observed, while a ratio around 2·0 was found in nuclei from tissues in which cells were predominantly in interphase.[18] Since the Ehrlich ascites tumor cells used in our experiments were harvested towards the end of their logarithmic growth period, the ratio close to unity found in the present investigation is in good agreement with the expectation based upon these observations.

The results indicate that nucleoproteins (DNP) extracted from Ehrlich ascites tumor cells contain –SH groups in a similar amount as rat liver nuclei in which Jellum and Eldjarn,[19] using a NaCl extraction method similar to ours, determined an –SH content of 60 μmoles/g protein.

The increase in the amount of the –SH groups in the DNP preparations from Ehrlich cells upon treatment with a disulphide reducing agent, indicates that also disulphide groups occur, amounting to about 50 per cent of the –SH groups. The proportion of the disulphides was less than 20 per cent in the histones prepared from thymus and regenerating liver.[20,21] The difference may be due, besides to a difference in the cellular material, also to the fact that in the latter case,[20] a lower concentration of denaturating agent (4 M urea) was used before the disulphide reduction than in our experiments. The lower concentration may have left some of the disulphide groups inaccessible for reduction, as suggested in a previous study.[12] Another explanation of the difference may be the possible difference in the stage of the generation cycle of regenerating liver cells and 5–6 days old Ehrlich ascites tumor cells. In sea urchin eggs, the proportion of –SH to the total –SH + SS content was shown to vary with the cell cycle from 0·2 in the mature unfertilized eggs to 0·59 just before cleavage.[22] Sadgopal and Bonner[23] have shown that the cysteine residues mainly occur in a reduced monomeric form in the histones of interphase chromosomes of HeLa cells while the sulphydryl groups are oxidized and the histones polymerized in metaphase chromosomes.

Chromatographic analysis of the acid extract of DNP after disulphide reduction revealed the presence of two –SH containing peptides one of which identified as glutathione (GSH). This suggests that DNP disulphide groups may be, at least in part, of the mixed type. The presence of thiol containing peptides bound to DNP as mixed disulphides has been shown by Ord and Stocken,[16,17] and Sakai and Dan[24] to exist also in sea urchin eggs. The nature of these mixed disulphides has not been established but Sakai and Dan concluded that the peptide they found is not GSH. Sadgopal and

Bonner[23] have also shown that metaphase chromosome contain non-histone, acid soluble proteins which are bound to the histones by disulphide bonds.

The special function of histone –SH and disulphide bonds has attracted much attention, but is still poorly understood. It is plausible that they play an important role in the events preceding mitosis.[6] This is also suggested by the variation in the thiol–disulphide ratio in DNP during the cell cycle with an oxidation of –SH groups and polymerization of histones before mitosis leading to condensation of the chromosomes.[23] Ord and Stocken[25] also showed that the metabolically active, diffuse chromatin contains a larger proportion of its sulphur in the thiol form than the metabolically inactive, dense chromatin. Thiol-blocking agents have also been shown to prolong the cell cycle or even prevent mitosis.[26]

Hilton and Stocken[4] reported that the oxidation of –SH groups in F3 histones increases the ability of these histones to repress DNA-dependent RNA synthesis. Thiol and disulphide groups of DNP may, thus, be of importance also in the regulation of gene activity.

The relatively large amount of thiol groups in histones, and the proximity of these thiols to DNA, renders them especially interesting with regard to their possible role in natural and/or artificial radiation protection. The result of the present study confirms the finding of Jellum and Eldjarn[19] that the radioprotective compound cysteamine readily binds with DNP. The decrease in the amount of free –SH groups after cysteamine treatment, and the release of about 80 per cent of the DNP-bound cysteamine upon reduction of the disulphide bonds, indicate that the major form of binding is by mixed disulphide formation, as has been suggested previously.[19] The remaining 20 per cent of cysteamine which could not be released, may be bound to DNP by some other type of binding, possibly by the formation of peptide bonds.

The results suggest, furthermore, that cysteamine also interacts with existing disulphide- and mixed disulphide-bonds. The –SH content of the acid soluble fraction of the cysteamine treated DNP-preparations was increased by 12 μmoles –SH/g of protein, as compared to the cysteamine-untreated preparations (Table 1). On the other hand, the amount of DNP-bound cysteamine was in the order of 18 μmoles/g protein (Table 2). The difference suggests that cysteamine, in addition to its binding to free –SH groups and forming new disulphide bonds, may also reduce pre-existing mixed disulphide bonds, and thereby release an equivalent amount of thiols (peptides?) which will be lost in connection with the isolation of DNP. This conclusion is further supported by the results of experiments in which cells pretreated with NEM were exposed to cysteamine. About 8 μmoles cysteamine/g of protein was bound to DNP in this case (Table 2), but, upon reduction, the amount of thiols in the acid extract increased by not more than about 2 μmoles/g.

In summary, the radioprotective compound cysteamine may affect DNP in the following ways: (a) it forms mixed disulphides with free –SH groups; (b) it reduces disulphide bonds, forms a mixed disulphide with one of the available –SH groups and releases acid soluble peptides in the process and; (c) it binds with DNP by formation of peptide bonds.

Littbrand and Révész[9] recently showed that cysteamine-treatment permits recovery of cells from sublethal radiation damage after irradiation under anoxic conditions, if oxygen is available within about half an hour. Without cysteamine, no recovery occurs after anoxic exposure even if oxygen is available shortly (within 1–2 min) after

irradiation. The authors postulate that the recoverable period, i.e. the time before radiation damage becomes irreversibly fixed, is limited and may last only a few minutes. Cysteamine is supposed to prolong the recoverable period. In view of the importance of DNP thiol and disulphide groups for cell replication and gene control, it is conceivable that the blockage of histone –SH groups or the reduction of the disulphide groups by cysteamine increases the repression of DNA activity and, thereby, prolongs the time-period before radiation-induced structural defects are replicated beyond possible repair. As an alternative explanation, it has been assumed[9] that fixation of the radiation damage is an enzymatic process which is slowed down by the action of cysteamine. The suggestion that cysteamine may act on the level of repair mechanisms has earlier also been proposed by Bacq and Goutier[27] and Révész.[28]

In earlier works[29,30] we reported that cysteamine releases protein-bound glutathione, and that this release may be of importance for the radio-protective effect of cysteamine. The finding that glutathione is bound to histones by mixed disulphide bonds in a close vicinity to the primary radiation target, i.e. DNA, and that cysteamine interacts with these bonds possibly releasing glutathione, further supports this idea.

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