Chemical Cross-Linking of Heteromeric Glucocorticoid Receptors†

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ABSTRACT: Glucocorticoid receptors of wild-type and nt^i ("increased nuclear transfer") mutant S49.1 mouse lymphoma cells exist in extracts under low-salt conditions predominantly as high molecular weight species ($M_r \geq 300\,000$). These receptor–hormone complexes are unable to bind to DNA. High salt (300 mM KCl) produces dissociated receptors of M_r 116 000 and 60-Å Stokes radius (wild type) and M_r 60 000 and 38-Å Stokes radius (nt^i mutant), both of which bind to DNA. We used reaction with bifunctional *N*-hydroxysuccinimide esters as well as oxidation with Cu^{2+}/*o*-phenanthroline to stabilize the high molecular weight structures. These cross-linked complexes do not interact with DNA, but reductive cleavage again produces the dissociable receptor forms and restores their ability to bind to DNA. The protein modifying reagents iodoacetamide and diethyl pyrocarbonate also produce stabilized high molecular weight receptor complexes. Cross-linking of the high molecular weight receptor forms can also be achieved in intact cells. Immunochemical techniques were used to prove that the complexes cross-linked either in vivo or in cell extracts do contain the heat shock protein of M_r 90 000 as a common constituent. The data show that the high molecular weight receptor complexes are preexisting in intact cells and that dissociation generates DNA binding ability.

Steroid hormone receptors may exist in different molecular forms in extracts of target cells, mainly depending on the conditions of temperature and ionic environment. The principal component in all of these structures is a polypeptide which carries a hormone binding domain and a DNA binding domain, both of which show interesting homologies among receptors for various steroids and of different species. Despite this common structural principle, the chain length varies considerably among different receptor types due to major differences in the amino-terminal parts [for a review, see Gehring (1987)]. High molecular weight receptor structures with M_r[1] 300 000 and above are detectable under conditions which avoid subunit dissociation [for reviews, see Sherman and Stevens (1984) and Vedeckis (1985)], and large glucocorticoid receptor complexes were found to contain only one steroid bearing polypeptide (Gehring & Arndt, 1985; Okret et al., 1985; Gehring et al., 1987) which is associated with other macromolecular components. The major such protein is the heat shock protein hsp90 which has been detected in high molecular weight steroid receptors of all hormone specificities analyzed in detail [for reviews, see Toft et al. (1987) and Pratt (1988)]. There is preliminary evidence for a dimer of hsp90 being associated with several steroid receptors (Renoir et al., 1984; Denis et al., 1987; Redeuilh et al., 1987; Arányi et al., 1988). In addition, a 59-kdalton protein was described as a component common to the high molecular weight forms of several receptor types (Tai et al., 1986), and RNA has also been found in association with glucocorticoid receptor complexes [for a review, see Webb and Litwack (1986)]. Recent evidence, however, suggests that endogenous tRNAs combine with glucocorticoid receptors subsequent to the dissociation of the high molecular weight complexes (Ali & Vedeckis, 1987).

The large heteromeric complexes of glucocorticoid receptors are known not to interact with DNA (Gehring et al., 1987; Pratt, 1988), but affinity for DNA develops upon subunit dissociation which leads to the exposure of the DNA binding domain that appears to be a preformed structure. Dissociation and thus activation to the DNA binding state may be achieved by warming receptor complexes or by exposing them to high ionic strength. Previous studies showed that certain transition metal oxyanions, in particular, molybdate, inhibit receptor dissociation by stabilizing the hsp90-containing complex [for a review, see Pratt (1988)]. Since, however, it has been suggested that the use of molybdate may produce artifactual receptor associations (Birmbaumer et al., 1984), we decided in the present investigation rather to study the large receptor structures in the absence of this anion. Instead, we used chemical cross-linking of subunits in high molecular weight glucocorticoid receptors in order to prevent them from separating in high-salt solutions. We obtained a clear correlation between dissociation and the evolvement of DNA binding ability. As in previous work we used wild-type and nt^i ("increased nuclear transfer") mutant receptors of mouse lymphoma cells. Even though the origin of the nt^i mutation has not been fully established, it is clear that the nt^i receptor contains a truncated steroid bearing polypeptide from which the amino-terminal part is missing (Gehring, 1987; Miesfeld et al., 1988). We now show that this domain of the wild-type receptor does not participate in cross-linking and is not involved in the stabilization of the high molecular weight heteromeric receptor structure.

Experimental Procedures

Chemicals. EGS and DSP were obtained from Pierce Chemical Co.; DEPC, iodoacetamide, and *o*-phenanthroline were purchased from Sigma.

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; DMSO, dimethyl sulfoxide; DSP, dithiobis(succinimidyl propionate); EDTA, ethylenediaminetetraacetic acid; EGS, ethylene glycol bis(succinimidyl succinate); hsp90, heat shock protein of M_r 90 000; IgG, immunoglobulin G; mab, monoclonal antibody; M_r, relative molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-ketal with acetone; Tris, tris(hydroxymethyl)aminomethane.

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Cell Culture. The S49.1 mouse lymphoma sublines S49.1G.3 (wild type) and S49.1TB.4.143R (nt^i) were used (Gehring & Hotz, 1983). Cells were grown and harvested as previously described (Gehring et al., 1987). The mab 49 producing hybridoma cells were grown in medium supplemented with 10% fetal calf serum.

Cell Extracts. Frozen cell pellets were homogenized as before (Gehring et al., 1987) except that 20 mM potassium phosphate buffer (pH 7.4 at 20 °C) containing 1 mM EDTA (homogenization buffer) was used and the final protein concentration was 10–15 mg/mL. Incubation with [^{3}H]triamcinolone acetonide (New England Nuclear, 1 TBq/mmol) was at 20 nM for 2 h at 0 °C; excess unbound hormone was removed with charcoal. Radioactivity was assessed by liquid scintillation spectrometry at 40% efficiency.

Protein Modifying Reactions. One milligram of EGS or DSP, dissolved in 10 μL of dimethyl sulfoxide, was added per milliliter of cell extract, and the mixture was incubated on ice for 30 min. Occasionally the solution turned turbid due to precipitation of some of the reagent; however, this did not markedly influence the reaction. Cross-linking was stopped by the addition of 30 μL of 3 M lysine/mL and further incubation for 20 min. *o*-Phenanthroline (final concentration 1.3 mM) and $CuSO_4$ (final concentration 0.25 mM) were added from a freshly prepared 10× stock solution, and incubation was at 20 °C for 30 min. Reaction with iodoacetamide (final concentration 20 mM) and DEPC (final concentration 70 mM) was at 0 °C for 30 min. Subsequent to these reactions KCl was added to a final concentration of 300 mM, and incubation was continued for 1 h at 0 °C in order to dissociate unreacted receptor complexes. Samples were then directly applied either to glycerol gradients or gel permeation columns. For use in DNA binding studies and DEAE-cellulose chromatography, samples were desalted by passage over Sephadex G-25 equilibrated in the above buffer.

Gel Permeation Chromatography. Gel filtration on Sephacryl S-300 (Pharmacia) was carried out as previously described (Gehring et al., 1987) in homogenization buffer containing 300 mM KCl and 10% glycerol. Routinely 250 000–1 000 000 dpm of protein-bound radioactivity was applied to the columns.

Sedimentation Analysis. Sedimentation in linear glycerol gradients was carried out as described (Gehring & Arndt, 1985) in 300 mM KCl containing buffer. Molecular weights were calculated from sedimentation coefficients and Stokes radii as before (Gehring et al., 1987).

DEAE-cellulose Chromatography. DEAE-cellulose columns of 10 mL (Whatman DE-52) were equilibrated with homogenization buffer. Desalted receptor preparations were applied and, after extensive rinsing with the above buffer containing 100 mM KCl, were eluted with buffer containing 300 mM KCl. Peak fractions were either directly submitted to gel filtration or incubated with mercaptoethanol.

DNA Binding Studies. DNA–cellulose columns of 2–3-mL bed volume were equilibrated in homogenization buffer. Desalted receptor preparations were applied, and the columns were incubated in the cold for 30 min. Unbound material was analyzed on gel permeation columns. In other experiments DNA binding ability of receptor preparations after cross-linking and cleavage was checked. For this purpose, pooled peak fractions from Sephacryl S-300 chromatography were concentrated by Centriflow ultrafiltration (Amicon), and the salt concentration was adjusted to 30 mM KCl. Free hormone dissociated from the receptors during these procedures was removed by charcoal treatment prior to application to DNA–cellulose. Bound material was eluted with 600 mM KCl, and radioactivity was measured.

Cleavage of Cross-Linked Receptors. Receptors cross-linked with DSP and chromatographed over DEAE-cellulose were diluted 1:1 with 50 mM Tris buffer (pH 8.5 at 20 °C) containing 1 mM EDTA and 300 mM KCl. Mercaptoethanol was added at 140 mM, and incubation was overnight at 0 °C under argon to prevent reoxidation. Receptors reacted with cupric *o*-phenanthroline were chromatographed over Sephacryl S-300, and peak fractions were pooled, concentrated by Centriflow (see above), and incubated with mercaptoethanol as above.

Cross-Linking of Receptors in Intact Cells. Cells were collected from cultures by centrifugation at 1000*g*, resuspended in phosphate-buffered saline at a density of about 5×10^8/mL and incubated with 20 nM [^{3}H]triamcinolone acetonide for 1 h at 0 °C. Cell suspensions were adjusted to 5% DMSO and incubated on ice with 2 mg of DSP/mL; after 60 min the reaction was stopped with lysine as above. Incubation with cupric *o*-phenanthroline was routinely in the absence of DMSO for 30 min at 20 °C at the same concentrations as used in the cell extracts. Cells were then washed twice with saline to remove free hormone and excess reagents. Trypan blue exclusion was used to check cell integrity, and >95% were found to be intact by this technique. Cell extracts were then prepared and analyzed as described above.

Purification of hsp90 and Production of Antiserum. The hsp90 was purified from wild-type S49.1 mouse lymphoma cells by DEAE-cellulose and hydroxylapatite column chromatography as described by Welch and Feramisco (1982). The protein was 90–95% pure as judged by SDS–PAGE and staining with Coomassie blue. The band of M_r 90 000 was cut out from unstained portions of the gels and electroeluted in a dialysis bag with Tris/glycine buffer. The solution was dialyzed against PBS and used for immunizing a New Zealand rabbit (Vaitukaitis, 1981). The antiserum detects on immunoblots mainly hsp90; however, two faint bands of $M_r \approx 63\,000$ and $\approx 47\,000$ were also seen when crude cell extracts were used.

Immunoaffinity Chromatography. Monoclonal antibody mab 49 was purified from hybridoma cell supernatants by protein A–Sepharose (Pharmacia) and coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Routinely 2–3 mg of pure IgG was coupled to 1 mL of Sepharose. After removal of free hormone, either cross-linked or untreated receptor samples were made 400 mM with respect to KCl and applied to 1-mL columns equilibrated with PBS. Binding of receptors was usually in the range of 80–90%. The columns were rotated end over end for 2 h in the cold and rinsed 6 times with 5 mL each of 20 mM phosphate buffer, pH 7.4, containing 600 mM KCl, followed by 5 more rinses with 1 mL of 0.5 M sodium thiocyanate, pH 7.5. Elution was with 3.5 M sodium thiocyanate, and 1-mL fractions were collected in tubes containing 50 μg of soybean trypsin inhibitor as a carrier protein. Peak fractions, as determined by radioactivity counting, were precipitated with trichloroacetic acid. Precipitated proteins were neutralized with 5 μL of 1 M NaOH and prepared for SDS–PAGE. Mercaptoethanol was added freshly at a concentration of 10% to each sample prior to boiling. This results in reductive cleavage of cross-linked complexes.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblotting. SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Protein bands were transferred to Immobilon PVDF membranes (Millipore) presoaked in methanol and then

Table I: Molecular Properties of Receptors[a]

receptor type	condition of analysis, [KCl] (nM)	modifying agents: DSP	modifying agents: Cu^{2+}/ o-phenanthroline	modifying agents: mercaptoethanol	Stokes radius (Å)	sedimentation coefficient (S)	molecular weight
wt	0	−	−	−	80.6[b]	9.6[b]	328 000
	300	−	−	−	59.9 ± 1.7	4.6 ± 0.3	116 000
	300	+	−	−	82.3 ± 2.9	9.1 ± 0.1	316 000
	300	+	−	+	60.2 ± 1.3		
	300	−	+	−	80.2 ± 2.6	9.9 ± 0.1	335 000
	300	−	+	+	61.6 ± 2.2		
nt^i	0	−	−	−	71.9[b]	9.8[b]	298 000
	300	−	−	−	38.4 ± 1.5	3.7 ± 0.1	60 000
	300	+	−	−	70.0 ± 1.6	9.4 ± 0.7	278 000
	300	+	−	+	38.5 ± 0.8		
	300	−	+	−	70.6 ± 2.2	10.1 ± 1.0	298 000
	300	−	+	+	37.8 ± 1.1		

[a] Stokes radii and sedimentation coefficients were determined in three to eight independent experiments; mean values and ranges are reported. [b] From Gehring et al. (1987).

equilibrated in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Transfer was carried out in a water-cooled blotting apparatus in the same buffer for 2 h at a constant voltage of 150 V. After transfer, protein bands were stained with 0.2% Ponceau S (Serva) in 3% trichloroacetic acid and 3% sulfosalycilic acid to visualize the marker proteins. Membrane lanes to be stained immunologically were cut out and destained with 50 mM Tris and 150 mM NaCl prior to blocking of nonspecific sites with 20% fetal calf serum in PBS containing 0.3% Tween 20 (Serva) for 1 h at room temperature. After a brief washing with PBS/Tween, membranes were incubated at 8 °C overnight either with a 1:1 mixture of blocking solution and hybridoma cell culture supernatant or with blocking solution containing rabbit serum at a concentration of 1 μL/mL. After an extensive washing with PBS/Tween, membranes were reincubated with blocking solution for 30 min at room temperature followed by the addition of horseradish peroxidase conjugated second antibodies (Sigma) at a concentration of 1:1000. Incubation was continued for a further 3 h at room temperature, and after repeated washings with PBS/Tween, membranes were developed with 4-chloro-1-napthol (Bio-Rad) as substrate (0.02% in 50 mM Tris-HCl, pH 7.5, containing 0.01% H_2O_2).

Results

Cross-Linking with Bifunctional Reagents. Wild-type and nt^i mutant glucocorticoid receptors of mouse lyphoma cells can be detected as large complexes of M_r 328 000 and 298 000, respectively, provided that the cell extracts are kept cold and that exposure to high ionic strength is avoided (Gehring et al., 1987). Treatment with high salt (300 mM KCl) in the cold or brief warming to 20 °C generates the dissociated receptor forms of M_r 116 000 and 60 000, respectively (Table I; Gehring et al., 1987). In order to prevent dissociation of the large receptor complexes, we used the chemical cross-linking reagents EGS and DSP. Both bifunctional compounds are *N*-hydroxysuccinimide esters which at pH 7 to 8 predominantly react with ε-amino groups of lysine residues in proteins (Lomant & Fairbanks, 1976; Abdella et al., 1979). The main difference between these compounds is that DSP contains a disulfide group which may be split by reduction while EGS is not cleavable under those conditions.

Figure 1 shows experiments in which wild-type and nt^i receptor complexes were reacted with EGS and subsequently analyzed in the presence of 300 mM KCl by gel permeation chromatography. In these samples only about half of the large receptor complexes dissociated to the lower molecular weight

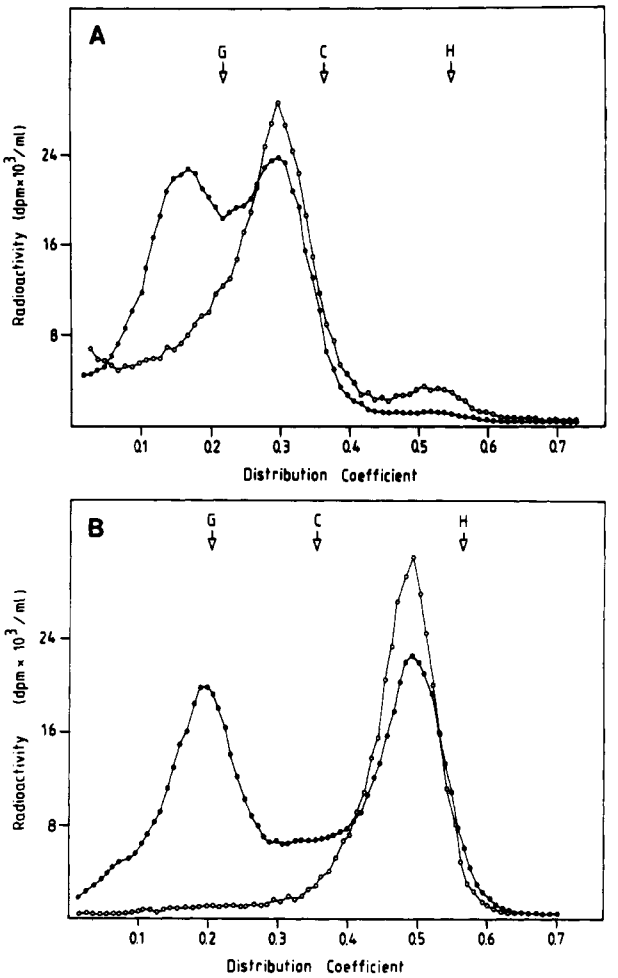

FIGURE 1: Gel filtration of EGS-treated receptors. Wild-type (A) and nt^i (B) receptor complexes with [^{3}H]triamcinolone acetonide were treated with EGS (●) or not (○) as described under Experimental Procedures. Marker proteins were *E. coli* β-galactosidase (G; R_s = 68.5 Å), bovine liver catalase (C; R_s = 52.3 Å), and human hemoglobin (H; R_s = 32.1 Å).

forms of about 60- and 38-Å Stokes radii, respectively. In the controls which were not treated with the cross-linking reagent, complete dissociation occurred in 300 mM salt. If cross-linking was carried out subsequent to exposure to high ionic strength, only the 60- and 38-Å peaks were obtained (data not shown).

The same results were obtained when DSP was used instead of EGS (Figure 2A,B). The large receptor complexes stabilized by covalent cross-linking had Stokes radii of 82.3 and

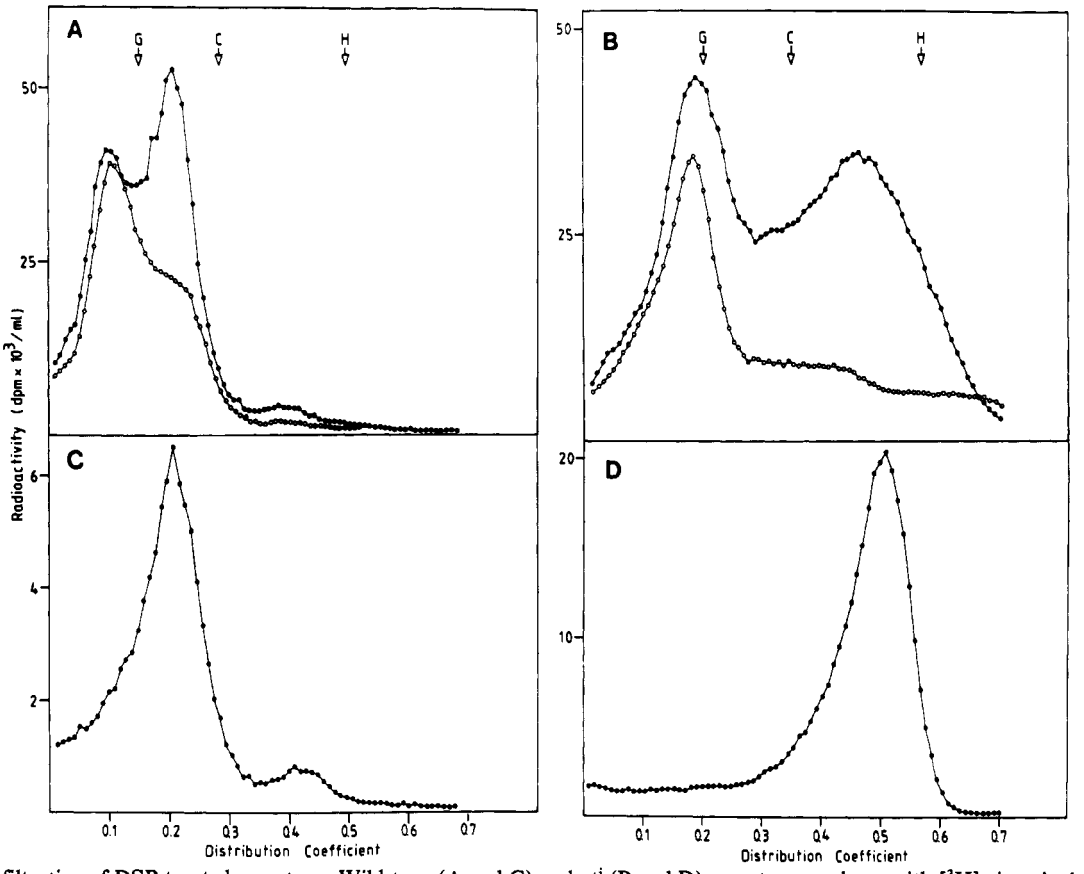

FIGURE 2: Gel filtration of DSP-treated receptors. Wild-type (A and C) and nt^i (B and D) receptor complexes with [^{3}H]triamcinolone acetonide were treated with DSP as described under Experimental Procedures and applied to columns either directly [A and B (●)] or after filtration over DNA–cellulose (○). In (C) and (D), the high molecular weight complexes were isolated by DEAE-cellulose chromatography, incubated with mercaptoethanol as described under Experimental Procedures, and submitted to gel filtration analysis. Markers were as in Figure 1.

70.0 Å and sedimentation coefficients of 9.1 S and 9.4 S, respectively, for the wild-type and nt^i receptors. These hydrodynamic parameters were used to compute the apparent molecular weights of the cross-linked receptor forms, and values of about 316 000 and 278 000 were obtained (Table I). In order to check the DNA binding properties of the cross-linked preparations, we used filtration through DNA–cellulose and analyzed the flow-through again by gel filtration. We now detected only the high molecular weight forms with Stokes radii of about 80 and 70 Å (Figure 2A,B, open symbols) while the activated receptor forms of about 60 and 38 Å were retained by DNA–cellulose. This proves that the cross-linked large receptor complexes of wild type and nt^i mutant do not bind to DNA. In this respect they behave identical with the unreacted receptors if dissociation is avoided (Gehring et al., 1987).

The above studies were carried out with receptor preparations which had been complexed with the steroid. In some experiments we reacted unliganded receptors with the bifunctional reagent DSP and subsequently incubated them with the radiolabeled hormone. We found that the cross-linking reaction resulted in substantial loss of steroid binding activity (about 90%). This instability is in contrast to previous observations with the chick oviduct progesterone receptor and various bis imidates as cross-linking reagents (Arányi et al., 1988).

Cleavage of Cross-Linked Receptors. In all our experiments cross-linking of receptor–hormone complexes with EGS and DSP was incomplete. For further experiments we therefore depended on separating the cross-linked complexes from the unreacted ones. This was easily achieved by use of DEAE-cellulose chromatography which in previous studies was shown to distinguish between activated and nonactivated wild-type and nt^i receptors (Dellweg et al., 1982; Gehring et al., 1987). The large receptor complexes stick quite tightly to the anion-exchange resin while the activated and dissociated receptors elute with a low salt concentration. Thus, receptor complexes treated with DSP were passed over DEAE-cellulose, the columns were rinsed with buffer containing 100 mM KCl, and the large receptor complexes were eluted with buffer containing 300 mM KCl. A sample of the cross-linked wild-type receptor indeed contained only the 80-Å material (data not shown). The eluates from DEAE-cellulose were incubated with mercaptoethanol overnight and submitted to gel permeation chromatography. As depicted in Figure 2C,D, the lower molecular weight complexes of 60.2 and 38.5 Å of wild-type and nt^i receptors were obtained at high yields (Table I). These complexes turned out to be able to interact with DNA as was shown by DNA–cellulose chromatography of pooled peak fractions. The receptors bound to DNA–cellulose to about the same extent as had previously been observed with unreacted activated complexes (Gehring, 1980; Gehring & Segnitz, 1988), i.e., about 45–50%.

Cross-Linking with Cupric o-Phenanthroline. Sulfhydryl groups in proteins can be linked to each other by mild oxidation. A convenient reagent is the complex of *o*-phenanthroline with cupric ion in conjunction with atmospheric oxygen (Kobashi, 1968; First & Taylor, 1984). As shown in Figure 3, treatment of wild-type and nt^i receptors with the Cu^{2+}/*o*-phenanthroline chelate resulted in complete cross-linking. The Stokes radii and sedimentation coefficients of these stabilized high molecular weight complexes were identical with those obtained for the native receptors (Table I). These oxidized complexes were unable to bind to DNA as was shown

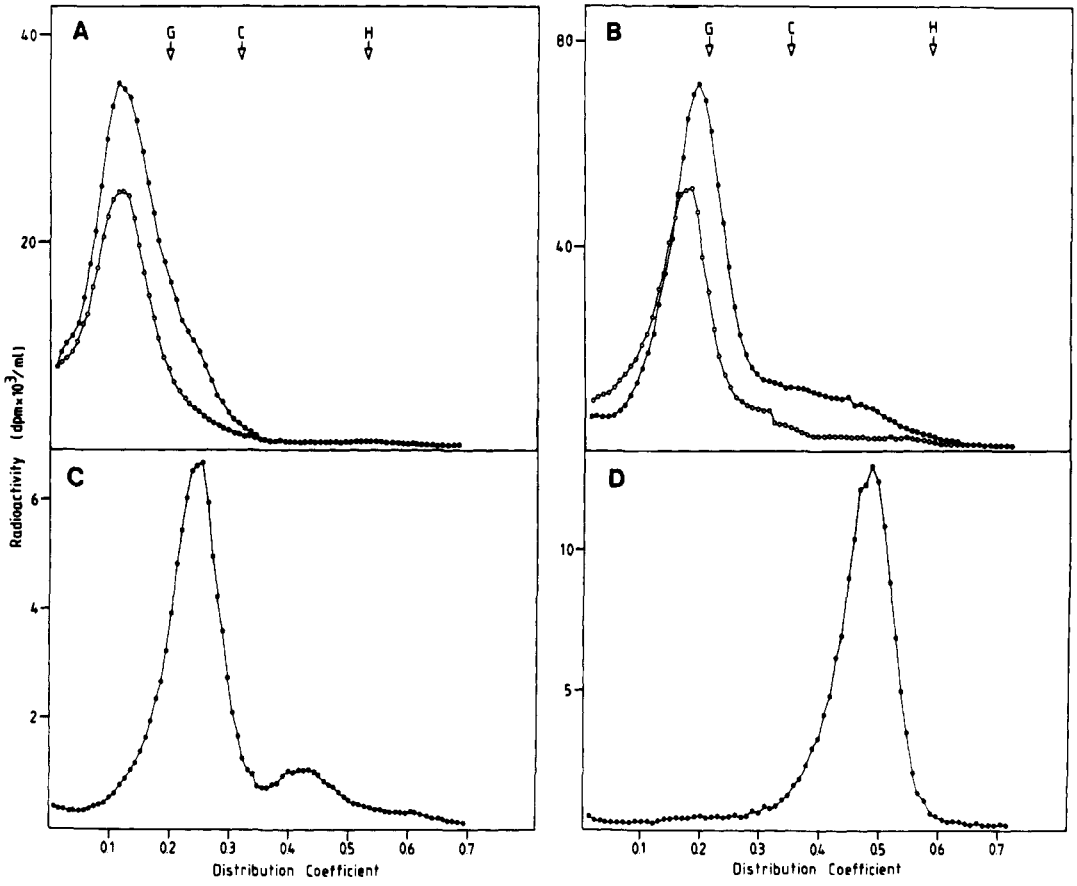

FIGURE 3: Gel filtration of receptors oxidized with cupric *o*-phenanthroline. Wild-type (A and C) and nt^i (B and D) receptor complexes with [^{3}H]triamcinolone acetonide were treated with Cu^{2+}/*o*-phenanthroline as described under Experimental Procedures and applied to the columns either directly [A and B (●)] or after filtration through DNA–cellulose (○). In (C) and (D), the oxidized complexes were incubated with mercaptoethanol as described under Experimental Procedures and submitted to gel filtration analysis. Markers were as in Figure 1.

by prior passage through DNA–cellulose (Figure 3A,B, open symbols). Reduction with mercaptoethanol again produced the receptor forms which fully dissociated in 300 mM KCl (Figure 3C,D; Table I). These cleaved receptors were found to bind to DNA–cellulose to about 50%.

Partial Proteolysis of Cross-Linked Wild-Type Receptors. In previous studies we showed that mild proteolysis with chymotrypsin or other proteases removes the amino-terminal domain from the wild-type receptor polypeptide and thus produces a receptor form which in many respects resembles the nt^i mutant receptor (Dellweg et al., 1982; Gehring & Hotz, 1983; Gehring, 1986; Gehring et al., 1987; Gehring & Segnitz, 1988). This part of the molecule was termed the "modulation domain" as its presence markedly influences the receptor's affinity to DNA. Chymotrypsin treatment was also found to remove the modulation domain from the high molecular weight form of the wild-type receptor, thereby creating a structure analogous to the large nt^i complex (Gehring & Arndt, 1985; Gehring et al., 1987). We now asked whether the modulation domain participates in cross-linking of the high molecular weight receptor structure when bifunctional *N*-hydroxy-succinimide esters or oxidation by Cu^{2+}/*o*-phenanthroline is being used.

Figure 4A shows an experiment with DSP. Chymotrypsin treatment of the cross-linked receptor produced a gel permeation profile (open circles) indistinguishable from that of the DSP-treated nt^i receptor (cf. Figure 2B) with peaks of 70.5- and 40.7-Å Stokes radii. The same elution profile was also obtained when the cross-linking reaction was carried out after incubation with chymotrypsin (not shown). Figure 4B shows the corresponding experiment with cupric *o*-phenanthroline. Again, protease treatment resulted in elution peaks of 69.2 and 40.3 Å which are characteristic for the nt^i receptor (cf. Table I).

Stabilization of High Molecular Weight Complexes by Other Agents. High molecular weight glucocorticoid receptors have been reported to contain RNA as one of their constituents [for a review, see Webb and Litwack (1986)], and irradiation with UV light has been used to cross-link the RNA moiety with the steroid-bearing polypeptide (Economidis & Rousseau, 1985). We therefore attempted to use UV irradiation to cross-link protein subunits in high molecular weight receptor complexes via RNA in much the same way as we used protein–protein cross-linking. In these experiments we included DEPC in the receptor preparations in order to inactivate ubiquitous RNases. We indeed obtained high molecular weight receptors which did not dissociate upon treatment with 300 mM salt. However, in control experiments in which UV irradiation was omitted we likewise observed such stabilized large complexes, while UV treatment in the absence of DEPC was ineffective. Figure 5 shows gel filtration experiments with wild-type and nt^i receptors which were reacted with DEPC; complete conversion to the undissociable complexes of 80.9 ± 1.5 Å (n = 3) and 70.4 ± 0.4 Å (n = 2) Stokes radii was attained.

An interesting result was obtained when the large wild-type complex was first oxidized with cupric *o*-phenanthroline and subsequently reacted with DEPC. In this case incubation with mercaptoethanol did not yield the dissociable receptor form. We therefore conclude that DEPC modifies amino acid side chains other than SH groups.

In other experiments we used the protein modifying reagent

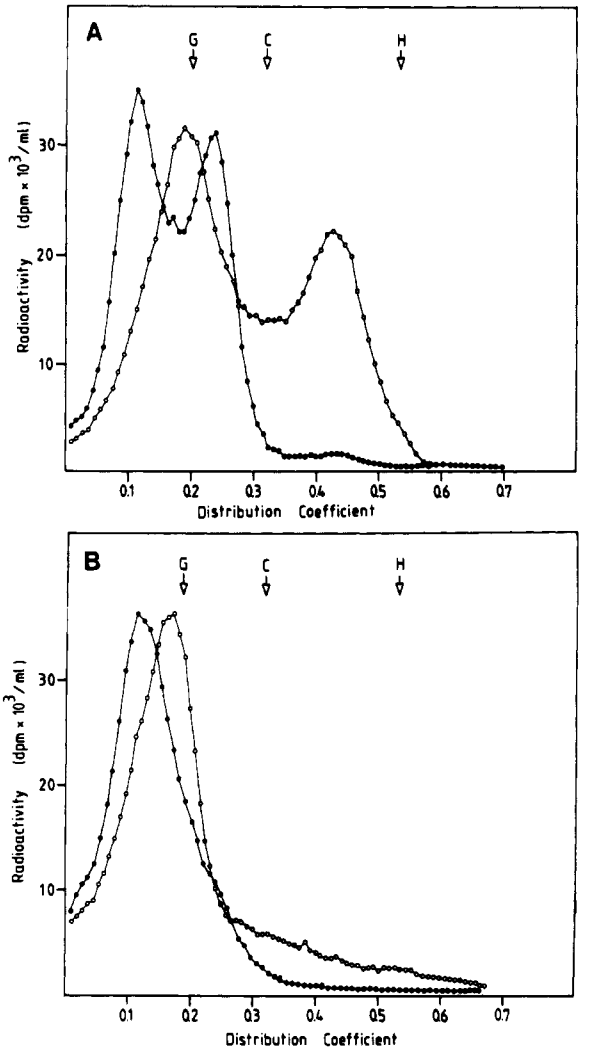

FIGURE 4: Chymotrypsin treatment of cross-linked receptors. Wild-type receptor complexes with [^{3}H]triamcinolone acetonide were reacted with either DSP (A) or Cu^{2+}/*o*-phenanthroline (B). Gel filtration was either prior to (●) or after a 15-min treatment with 10 μg/mL bovine α-chymotrypsin at 0 °C (○). Proteolysis was stopped by the addition of 1 mM phenylmethanesulfonyl fluoride. Markers were as in Figure 1.

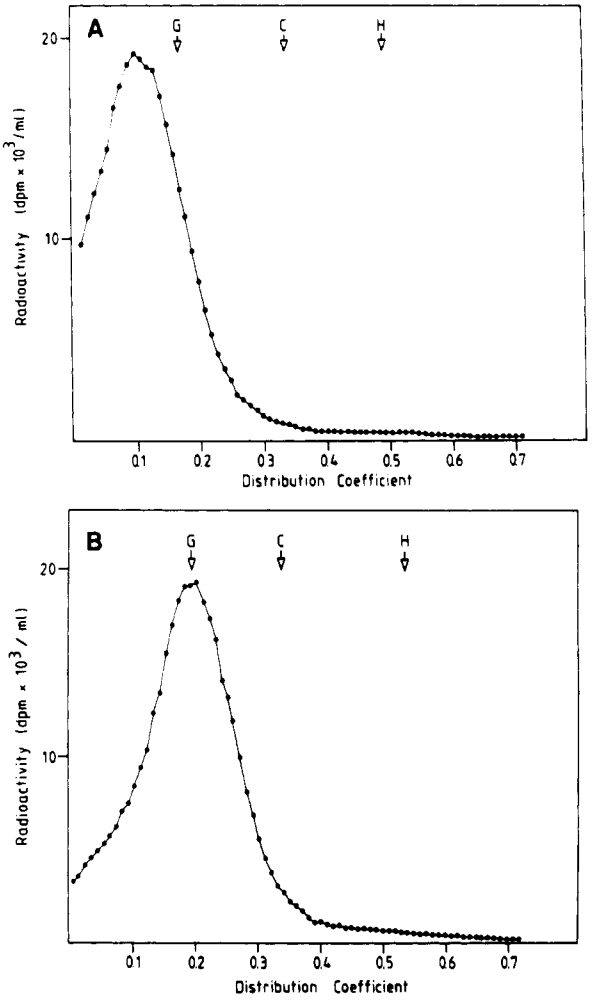

FIGURE 5: Gel filtration of receptors treated with DEPC. Wild-type (A) and nt^i (B) receptor complexes with [^{3}H]triamcinolone acetonide were treated with DEPC as described under Experimental Procedures and directly submitted to gel filtration. Markers were as in Figure 1.

iodoacetamide which is known to preferentially alkylate sulfhydryl groups of cysteine residues. Upon gel permeation chromatography we again detected large-sized receptor complexes which did not dissociate in 300 mM KCl. Stokes radii of 75.0 ± 2.6 Å (n = 3) and 68.5 ± 1.6 Å (n = 4) were obtained with wild-type and nt^i receptors, respectively. In the case of the wild type the Stokes radius is somewhat smaller than that observed in the experiments with bifunctional reagents and cupric *o*-phenanthroline (cf. Table I). The significance of this difference is not clear; however, suggestive evidence for receptor forms of intermediate sizes has previously been obtained (Gehring et al., 1987).

Cross-Linking of Receptors in Intact Cells. In order to find out whether high molecular weight receptor complexes are preexisting in cells, we used the following approach. Cells pretreated with the radiolabeled hormone in the cold were reacted with the cross-linker DSP in the presence of DMSO. Following incubation with excess lysine and extensive washing, the cells were homogenized, and the extracts were submitted to gel permeation chromatography under high-salt conditions. The stabilized complexes of 80 and 70 Å were indeed observed with wild-type and nt^i mutant cells (Figure 6). Cleavage of these cross-linked receptors was again attained by incubation with mercaptoethanol. As described above, we separated the cross-linked from the unreacted complexes by chromatography on DEAE-cellulose prior to reductive cleavage. Gel permeation chromatography under high-salt conditions produced receptor peaks with Stokes radii of about 60 and 38 Å, respectively, for wild-type and nt^i receptors (data not shown).

In a control experiment we switched the incubations with the cross-linker and with lysine. In this case no stabilized large complexes were obtained, showing that the cross-linking reaction takes place in intact cells rather than after cell disruption. Moreover, the yields of cross-linked receptor species depended largely on the presence of DMSO, which is known to facilitate membrane passage.

Oxidation of receptor complexes with Cu^{2+}/*o*-phenanthroline may also be accomplished in intact cells. Again, the large structures of about 80 and 70 Å of wild-type and nt^i receptors, respectively, were obtained (data not shown). However, the yields of the cross-linking turned out to be quite variable. Incubation at 20 °C is seriously hampered by the fact that activation proceeds simultaneously with cross-linking. On the other hand, we never observed any cross-linking in intact cells at 0 or 10 °C with Cu^{2+}/*o*-phenanthroline.

hsp90 as a Component of Cross-Linked Receptors. The following experiment was designed in order to ascertain the identity of the large receptor complexes cross-linked in whole cells and in cell extracts. As pointed out in the introduction, hsp90 has been identified as a component of high molecular

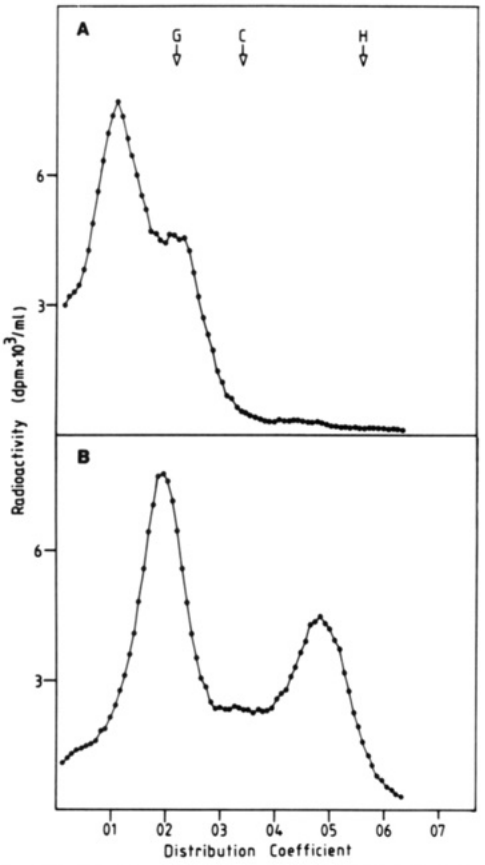

FIGURE 6: Gel filtration of receptors cross-linked in intact cells. Wild-type (A) and nt^i mutant (B) S49.1 mouse lymphoma cells were incubated with [^{3}H]triamcinolone acetonide and subsequently treated with DSP as described under Experimental Procedures. Cell extracts were analyzed by gel permeation chromatography. Markers were as in Figure 1.

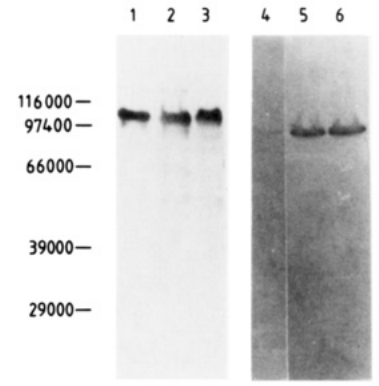

FIGURE 7: Immunoblot analysis of immunoaffinity-purified wild-type receptor complexes. Samples of either untreated receptor–[^{3}H]-triamcinolone acetonide complexes (lanes 1 and 4) or receptor complexes reacted with DSP in cell extracts (lanes 2 and 5) or intact cells (lanes 3 and 6) were purified on mab 49–Sepharose columns as described under Experimental Procedures. Receptor-containing fractions equivalent to 300 000 dpm (lanes 1–3) or 750 000 dpm (lanes 4–6) of bound hormone were submitted to SDS–PAGE. Proteins were transferrd to Immobilon PVDF membranes and incubated either with the receptor-specific monoclonal antibody map 49 (lanes 1–3) or with antiserum against the mouse hsp90 (lanes 4–6) as described under Experimental Procedures. Molecular weight markers were *E. coli* β-galactosidase (116 000), rabbit muscle phosphorylase *b* (97 400), bovine serum albumin (66 000), rabbit muscle aldolase (39 000), and bovine carbonic anhydrase (29 000).

weight receptor forms. For detecting this protein in cross-linked complexes, we used a specific antiserum raised against mouse hsp90. Since hsp90 is a very abundant cellular protein, we had to apply a receptor-specific purification method and decided to use immunoaffinity chromatography with the monoclonal antibody mab 49 (Westphal et al., 1982) coupled to Sepharose. This antibody is known to recognize an epitope within the steroid binding receptor polypeptide which is absent from the nt^i mutant receptor (Westphal et al., 1984). Figure 7 shows the presence of hsp90 in wild-type receptor complexes cross-linked with DSP either in a cell extract (lane 5) or in intact cells (lane 6). However, hsp90 was not detected in a control experiment in which cross-linking was omitted (lane 4). Washing of the immunoaffinity column with 0.5 M thiocyanate turned out to be essential for removing noncovalently bound hsp90, which otherwise nonspecifically sticks to the column. The results prove that the high molecular weight receptor complexes in intact cells and in cell extracts do contain hsp90. In an additional experiment we found that the wild-type receptor cross-linked in cell extracts by oxidation with cupric *o*-phenanthroline likewise contains hsp90 (data not shown).

Discussion

Chemical cross-linking of amino acid side chains of proteins has become a very useful technique for studying the quarternary structure of oligomeric proteins (Peters & Richards, 1977). The method, of course, depends on the availability of reactive target groups on neighboring protein subunits in appropriate molecular spacing and geometry. In a previous study with glucocorticoid receptors, Arányi (1984) used bis imidates of varying chain length and obtained complete cross-linking of the large receptor form by use of dimethyl sebacimidate. In the present study we used bifunctional symmetrical *N*-hydroxy esters which like the imidates react with ϵ-amino groups of lysine residues to form cross-links (Lomant & Fairbanks, 1976; Abdella et al., 1979). The reagents EGS and DSP produced high molecular weight forms of wild-type and nt^i mutant receptors which did not dissociate upon treatment with high ionic strength buffers. These receptor forms stabilized by covalent cross-linking were unable to bind to DNA as has previously been observed by use of bis imidates (Arányi, 1984). Our main interest, however, was to use cross-linking reagents which allow subsequent reversal. Thus, the disulfide contained in DSP may be cleaved by treatment with mercaptoethanol. Cleavage of the stabilized large receptor complexes followed by treatment with high salt again led to lower molecular weight receptor forms of the wild type and the nt^i mutant, i.e., $M_r \approx 115\,000$ and $\approx 60\,000$, respectively. These receptors regained the ability to interact with DNA. The data clearly demonstrate that chemical cross-linking of the heteromeric receptor structures precludes activation to the DNA binding forms. Thus activation is necessarily accompanied by dissociation of the large receptor structures, and it is likely that activation solely involves subunit dissociation.

This conclusion is corroborated by our findings with a mild oxidant. The Cu^{2+}/*o*-phenanthroline chelate produced under aerobic conditions stabilized high molecular weight receptor complexes presumably by interchain disulfide bonding. Reductive cleavage of such S–S bridges again allowed dissociation and hence activation to DNA binding ability. In similar experiments with androgen receptors, Wilson et al. (1986) have also observed the stabilization of a high molecular weight complex upon treatment with cupric *o*-phenanthroline. With hydrogen peroxide as oxidant, the rat liver glucocorticoid recpetor was prevented from dissociating to the DNA binding form, and reduction with dithiothreitol restored its ability to dissociate (Tienrungroj et al., 1987; Bresnick et al., 1988).

These observations suggest that certain cysteine residues in the large receptor structures need to be present in the reduced state in order for dissociation to occur. Moreover, disulfide bond reduction or thiol–disulfide exchange might also be involved in the mechanism of activation of receptor complexes not previously exposed to any chemical reagent. Evidence for disulfide reduction has in fact been reported for the androgen receptor (Wilson et al., 1986). In the present study we used iodoacetamide as a sulfhydryl blocking agent. This compound has previously been shown to inhibit glucocorticoid receptor binding to DNA (Young et al., 1975); we now demonstrate that it prevents large receptor complexes from dissociating in high-salt buffers. This stabilizing effect on heteromeric receptor structures may be due to blocking of essential SH groups on the receptors themselves as well as sulfhydryls on endogenous cellular reducing agents which possibly participate in disulfide exchange reactions. On the other hand, it recently became clear that unactivated glucocorticoid receptors contain thiol groups which may be modified, for example, by methyl methanethiosulfonate without impairment of dissociation (Tienrungroj et al., 1987).

The compound DEPC is widely used as a protein modifying reagent which preferentially reacts with histidines and thus inactivates enzymatic activities in which the imidazole ring is involved. However, carbethoxylation of other nucleophilic groups of amino acid side chains like SH groups may also occur (Miles, 1977). Moreover, cross-linking of multimeric protein structures upon DEPC treatment has been observed in some instances (Wolf et al., 1970; Steck, 1972); it has been suggested that intermolecular peptide-like bonds involving ϵ-amino groups of lysine and carboxylic groups of aspartic and glutamic acid may be formed (Wolf et al., 1970). On the other hand, modification of amino acid groups like histidyl residues (Schirmer et al., 1970) may well exert secondary effects on the subunit arrangement of protein structures. In the case of high molecular weight glucocorticoid receptors, it is not clear which amino acid groups are the targets for reaction with DEPC, but it appears unlikely from our experiments that SH groups are involved. It remains to be established whether stabilization of the large structures occurs by cross-linking of subunits or by some other mechanisms.

The position of heat shock protein of M_r 90000 in relation to the steroid binding polypeptide is an important problem. The fact that the modulation domain can be cleaved off by proteolysis from cross-linked wild-type receptors proves that it does not participate in the cross-linking reactions between subunits and thus appears to have no direct contact to hsp90. The association of hsp90 with the receptor polypeptide chain is probably mediated by some region within the hormone binding domain which shows homology among several steroid hormone receptors (Danielsen et al., 1987; Pratt et al., 1988).

A hitherto unresolved problem was whether the association of steroid binding polypeptides with other components like hsp90 is a normal cellular entity or whether association occurs artifactually as a consequence of cell homogenization. We now present unequivocal evidence for high molecular weight receptor complexes existing in intact cells. We were able to carry out cross-linking reactions in unruptured cells and detected stabilized high molecular weight structures with the same molecular properties. Moreover, the receptors cross-linked in intact cells and in cell extracts were shown to contain hsp90 as a common constituent. Taken together, this suggests that the complexes are indeed identical. Our observations clearly support the view that activation of receptor–glucocorticoid complexes may occur in intact cells under physiological conditions (Munck & Foley, 1979; Markovič & Litwack, 1980) and that this involves dissociation.

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Accessibility and Mobility of Lysine Residues in β-Lactoglobulin

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ABSTRACT: N^{ϵ}-[$^2H_6$]Isopropyllysyl-β-lactoglobulin was prepared by reductive alkylation of β-lactoglobulin with [$^2H_6$]acetone and $NaBH_4$ to provide a ^2H (NMR) probe for the study of lysine involvement in lipid–protein interactions. Amino acid analysis showed 80% of the protein's 15 lysine residues to be labeled. Unmodified lysine residues were located through peptide maps produced from CNBr, tryptic, and chymotryptic digests of the labeled protein. Lys_{47} was not modified; $Lys_{135,138,141}$, located along an amphipathic helical rod, were each partially unmodified. All other lysine residues were at least 90% modified. Average correlation times calculated from ^2H NMR spectra were 20 and 320 ps for 8.7 and 3.3 residues, respectively, in 6 M guanidine hydrochloride; in nondenaturing solution, values of 70 and 320 ps were obtained for 6.5 and 3.2 residues, respectively, with the remaining 2.3 modified residues not observed, suggesting that side chains of lysine residues in unordered or flexible regions were more mobile than those in stable periodic structures. ^2H NMR spectra of the protein complexed with dipalmitoylphosphatidylcholine confirmed the extrinsic membrane protein type behavior of β-lactoglobulin previously reported from ^{31}P NMR studies of the phospholipids complexed with β-lactoglobulin. Although no physiological function has yet been identified, comparison of these results with the X-ray structure [Papiz et al. (1986) *Nature (London) 324*, 383–385] supports the hypothesis that residues not accessible for modification may help to stabilize the cone-shaped β-barrel thought to contain binding sites for small lipid-soluble molecules.

β-Lactoglobulin (β-lg),[1] the major whey protein of bovine milk, has been used extensively as a model for the study of the interactions of globular proteins. Its interactions in vitro include complex formation with retinol (Hemley et al., 1979; Fugate & Song, 1980) and other aromatics (Farrell et al., 1987) and specific binding of sodium dodecyl sulfate (Jones & Wilkinson, 1976; Hillquist-Damon & Kresheck, 1982), alkanes (Wishnia & Pinder, 1966), and guanidinium ions (Pace & Marshall, 1980). Binding of β-lg to triglycerides (Smith et al., 1983) and phospholipids (Brown et al., 1983) in a manner similar to that of the peripheral membrane protein myelin (Fraser & Deber, 1984) has also been reported. Recent comparisons which show partial sequence identity (Pervaiz & Brew, 1985; Godovac-Zimmermann et al., 1985) and similar crystal structure (Papiz et al., 1986; Sawyer et al., 1985; Newcomer et al., 1984) with human retinol binding protein suggest that β-lg may have had a role in ruminant nutrition as a carrier of small, lipid-soluble molecules such as vitamin

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[1] Abbreviations: β-lg, β-lactoglobulin; RCM-β-lg, disulfide-reduced, S-carboxymethylated β-lg; Ip-β-lg, isopropyllysyl-β-lg; RCM-Ip-β-lg, reduced, S-carboxymethylated Ip-β-lg; Ip-RCM-β-lg, reductively alkylated after reduction and S-carboxymethylation; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; TPCK-trypsin, trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; CNBr I, cyanogen bromide fragment of β-lg which eluted first on gel permeation chromatography; Ip CNBr I, cyanogen bromide fragment of Ip-β-lg which eluted first on gel permeation chromatography.