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# EFFECT OF CROSS-LINKING ON GLUCOCORTICOID RECEPTOR ACTIVATION

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Chick thymus cytosol glucocorticoid receptor complexed with [$^3$H]-triamcinolone acetonide was heat-activated after treatment with diimidates of varying chain length. Diimidates with maximal effective reagent length shorter than 0.5 nm influenced DNA-cellulose binding only slightly, whereas sebacic diimidate (maximal effective reagent length 1.21 nm) blocked activation completely. Coupled activation - deactivation process induced in the presence of calcium was again inhibited only by the longer diimidates. Sebacic diimidate treated complex had a molecular mass similar to that of the molybdate stabilized nonactivated complex, as judged by gel permeation chromatography. It is suggested that prevention of size reduction by cross-linking blocks receptor activation as well.

Activation of the hormone-receptor complex, i.e. its change into a form capable of binding to DNA or nuclei, appears to be a necessary step in glucocorticoid hormone action (1). Activation is promoted *in vitro* by elevated temperatures and/or ionic strength (2,3), by gel filtration or dilution (4).

The process of activation results in changes in pI, surface charges and hydrophobicity (1) and a reduction in the size of the complex (5,6). It has been shown that blocking a lysine residue(s) by pyridoxal 5'-phosphate prevents activation of the complex (7,8).

Here we report on the use of lysine specific cross-linking reagents, diimidates, in the further analysis of the structural changes giving rise to the activated complex.

## MATERIALS AND METHODS

Chemicals. [1,2-$^3$H]-triamcinolone acetonide (26 Ci/mmol) was obtained from the Radiochemical Centre Amersham, U.K. Diimidates were prepared by the procedure of Mc Elvain and

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Schroeder (9). Norit A, Triton X-100 and dithiothreitol were from Serva, bovine serum albumin from Sigma, cellulose CF 11 from Whatman Ltd., and Sephacryl S-200 (superfine) from Pharmacia. DNA, purified from chicken blood, and all other chemicals were of reagent grade and were from Reanal, Hungary. DNA was coupled to cellulose by the procedure of Alberts and Herrick (10).

Preparation of cytosol. Six- to nine-week old Hunnia hybrid chickens were used. Thymus cytosol was prepared as described (11) except that 0.2 M triethanolamine-HCl buffer, pH 8.0 containing 1.0 mM EDTA and 2 mM dithiothreitol was used as homogenizing buffer.

Treatment with cross-linking agents. Cytosol was mixed with an equal volume of $^3$H-triamcinolone acetonide (40 nM) dissolved in the homogenizing buffer. The mixture was incubated at 0°C for 2.5 hours. Then a diimidate (final concentration 10 mM) or methyl acetimidate (final concentration 20 mM) was added and the incubation continued at 10°C for 30 min. Cross-linking was complete under these conditions (12).

Miscellaneous. Triamcinolone acetonide binding assay, Sephacryl chromatography and DNA-cellulose binding assay were performed as described (11). Protein content was determined by the Coomassie brilliant blue method (13) using bovine serum albumin as standard. Radioactivity was measured in a Beckman LS 350 radiospectrofluorimeter with 30% efficiency.

## RESULTS AND DISCUSSION

Cytosol glucocorticoid receptor complexed with triamcinolone acetonide acquired the ability to bind to DNA-cellulose upon incubation at 25°C within a few minutes. Neither kinetics of activation nor the maximum activation attained was significantly influenced by treatment with methyl acetimidate, malonic diimidate or succinic diimidate. However, adipic diimidate, and sebacic diimidate reduced DNA-cellulose binding very strongly (Fig. 1).

We have found recently that chick thymus glucocorticoid-receptor complex undergoes a relatively fast activation in the presence of $Ca^{2+}$ ions which is rapidly followed by deactivation, i.e. irreversible loss of affinity for DNA (14). Diimidates of short reagent length and methyl acetimidate had again only a moderate effect on the coupled activation deactivation process, but sebacic diimidate blocked activation completely (Fig. 2).

Diimidates are able to cross-link only lysine residues whose reactive ε-amino groups are closer to each other than the maximal

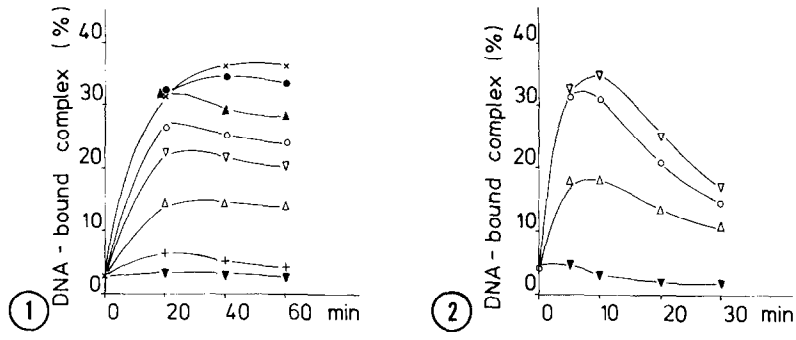


Fig. 1. Activation of the cross-linked receptor-ligand complexes. Chick thymus glucocorticoid receptor - triamcinolone acetonide complex was cross-linked with malonic diimidate (▽-▽); Succinic diimidate (●-●); glutaric diimidate (▲-▲); adipic diimidate (△-△); suberic diimidate (+-+); or sebacic diimidate (▼-▼). The non-cross-linked control was reacted with methyl acetimidate (○-○) or was given buffer only (×-×). Activation was performed at 25°C for the indicated times and DNA-cellulose binding was determined thereafter. DNA-bound receptor is expressed as percent of the total triamcinolone acetonide-receptor complex formed.

Fig. 2. Activation and deactivation of the cross-linked complex. Receptor-triamcinolone acetonide complex was treated with methyl acetimidate (○-○); malonic diimidate (▽-▽); adipic diimidate (△-△) or sebacic diimidate (▼-▼). Activation - deactivation cycle was induced by incubation at 25°C in the presence of 4 mM $CaCl_2$.

effective length of the diimidate reagent (12). This allowed us to measure the distance between the side chains whose cross-linking prevented activation.

To this end in Fig. 3 we replotted the 40 min data of Fig. 1 as a function of the maximal effective chain length of the diimidates. The distance diagram shows that about 0.7 nm reagent

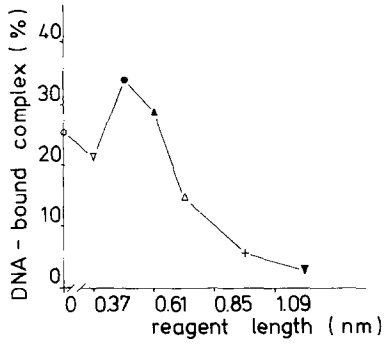


Fig. 3. Distance diagram. The 40 min values of Fig. 1 were plotted as a function of the maximal effective reagent length. The symbols correspond to those of Fig. 1. The line beteen the symbols has no physical meaning, it only serves illustrative purposes.

length was required for diimidates to be effective in blocking activation. This distance is much shorter than the Stokes radius of even the smallest receptor fragment (1.4 nm) that still binds hormone (15). It seems very probable that the two lysines involved are within a single polypeptide chain or are residing in two neighbouring subunits of the receptor molecule.

We examined therefore, if sebacic diimidate treatment of the complex effected its size. Gel permeation chromatographic analysis revealed that after cross-linking the apparent molecular mass of the complex was similar to that of the molybdate stabilized non-activated complex. On the other hand, the complex seemed smaller by Sephacryl chromatography if it was neither stabilized by molybdate ions nor cross-linked by sebacic diimidate. However, the overoll size distribution pattern was not significantly affected by sebacic diimidate, as judged from the 280 nm absorbance values (Fig. 4). This votes against the possibility that cross-linking by sebacic diimidate would glue cytosol

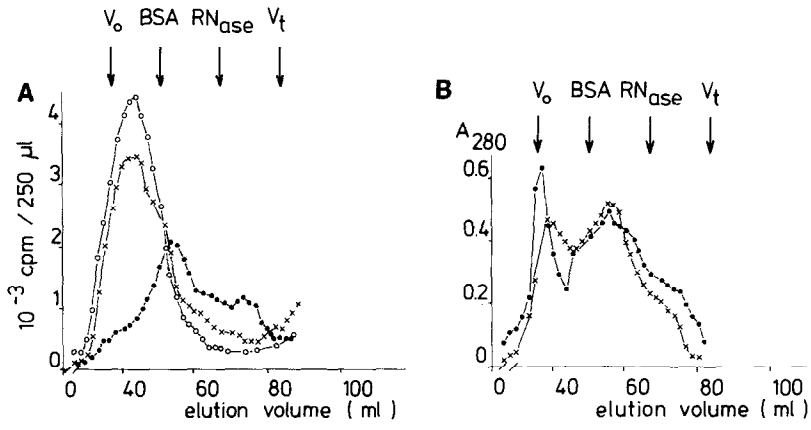


Fig. 4. Gel permeation chromatographic analysis of the cross-linked complex. Cytosol receptor was prepared and complexed with $^3$H-triamcinolone acetonide in the presence ( ○-○ ) or in the absence ( ×-×, ○-○ ) of 20 mM molybdate. The complex was analysed by gel permeation chromatography directly ( ○-○, ●-● ) or after cross-linking with sebacic diimidate ( ×-× ). A: Radioactivity (cpm/fraction) B: absorbance at 280 nm. The arrows show the elution volumes of Dextran blue, bovine serum albumin, ribonuclease A and $^3$H-methionine, respectively.

constituents together in a nonspecific manner under our conditions. It has been recently reported that activation of the glucocorticoid receptor protein was accompanied by fragmentation of the complex or dissociation into subunits (5,6,16). Our results demonstrate that activation can be prevented by cross-linking the nonactivated glucocorticoid receptor complex and thereby blocking separation of the subunits or receptor fragments. It thus seems very probable that size reduction of the nonactivated complex is not only coincidental with, but is also necessary for its activation.

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REFERENCES

1. Schmidt, T.J. and Litwack, G. (1982) Phys. Rev. 62, 1131-1192.
2. Milgrom, E., Atger, M. and Baulieu, E-E. (1973) Biochemistry 12, 5198-5205.
3. Kalimi, M., Colman, P. and Feigelson, Ph. (1975) J. Biol. Chem. 250, 1080-1086.
4. Higgins, S.J. Rousseau, G.G., Baxter, J.D. and Tomkins, G.M. (1973) J. Biol. Chem. 248, 5866-5872.
5. Holbrook, N.J., Bodwell, J.E., Jeffries, M. and Munck, A. (1983) J. Biol. Chem. 258, 6477-6485.
6. Middlebrook, J.L. and Aronow, L. (1977) Endocrinology 100, 271-282.
7. Cake, M.H., Disorbo, D.M. and Litwack, G. (1978) J. Biol. Chem. 253, 4886-4891.
8. Cidlowski, J.A. and Thanassi, J.W. (1978) Biochem. Biophys. Res. Commun. 82, 1140-1146.
9. McElvain, S.M. and Schroeder, J.P. (1949) J. Am. Chem. Soc. 71, 40-46.
10. Alberts, B. and Herrick, G. (1971) In: Methods in Enzymology (Ed. L. Grossman and K. Moldave) Acad. Press New York, London, Vol. XXI, pp. 198-217.
11. Arányi, P. and Náray, A. (1980) J. Steroid Biochem. 12, 267-272.
12. Hajdu, J., Dombrádi, V., Bot, Gy. and Friedrich, P. (1979) Biochemistry 18, 4037-4041.
13. Spector, T. (1978) Anal. Biochem. 86, 142-146.
14. Arányi, P. (1983) Eur. J. Biochem. 129, 549-554.
15. Ninh, N.V., Arányi, P. and Horváth, I. (1982) J. Steroid Biochem. 17, 599-601.
16. Vedeckis, W.V. (1983) Biochemistry 22, 1983-1989.