Modulation of Acetylcholine Receptor States by Thiol Modification†

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ABSTRACT: Chemical modification of thiol groups in acetylcholine receptor rich membranes from *Torpedo* gives rise to changes in receptor affinities, in agonist-induced transitions, and in the physical state of the receptor itself. The changes are dependent on the nature of the cholinergic ligand and on the thiol modification involved. Oxidation or alkylation without prior reduction of the free sulfhydryls is without effect on the above receptor properties as judged by α-toxin association rate measurements in the presence of full or partial agonists or antagonists. Dithiothreitol reduction diminishes the inhibitory capacity exerted by typical agonists on apparent toxin association rates and generates receptor states with affinities lower than those of normal membranes. Alkylation of the reduced thiols diminishes the affinity even more, shifting the equilibrium to "ultralow" forms. Antagonist responses are not affected by any of these treatments within the resolution of the method. Thiol modification has a complex effect on the intrinsic fluorescence of the receptor membranes. All thiol reagents cause, by themselves, similar patterns of this property, though saturation of the perturbation is achieved at different concentrations. Thiol-modified membranes respond in an altered manner to cholinergic ligands. In full agreement with the results from toxin kinetics, oxidizing and mild alkylating reagents do not affect cholinergic responses, but both reduction and reduction plus alkylation profoundly modify the fluorescence changes induced by cholinergic ligands. Since affinity labeling of selectively reduced receptor with the alkylating agent [4-(*N*-maleimido)phenyl]trimethylammonium (MPTA) produces essentially the same alterations, it is suggested that the disulfide bond in the vicinity of the receptor recognition site is affected by these treatments and is mainly responsible for the altered ligand discrimination and the formation of modified receptor states. The transformation of the agonist-like into antagonist-like response to a bis-quarternary partial agonist after reduction is the extreme case of these alterations. A hypothesis is presented on the modulation of partial agonism (mixed agonist and local anesthetic-like activity) by the thiol redox state. The occurrence of membrane-bound acetylcholine receptor states having ultralow affinity for agonists is discussed in relation to the putative native state of the receptor under physiological conditions.

The involvement of sulfur-containing amino acid residues in the structure and function of the acetylcholine receptor (AcChR)[1] is well documented. On the one hand the studies of Karlin and co-workers made it possible to chemically dissect a particular disulfide bond in the vicinity of the AcChR recognition site, which upon reduction renders a highly reactive sulfhydryl group available for specific acylation or alkylation with affinity labels both in vivo (Karlin & Winnik, 1968; Karlin, 1969; Silman & Karlin, 1969; Mittag & Tormay, 1970; Brown & Kwiatkowski, 1976) and in vitro [Karlin & Cowburn, 1974; Weill et al., 1974; Barrantes et al., 1975; Froehner et al., 1977; Damle et al., 1978; Sobel et al., 1977; see reviews in Karlin et al. (1976) and Karlin (1977)]. The "active-site-directed" probes have enabled the quantitation of AcChR sites in intact cells (Karlin et al., 1971), in membrane fragments (Karlin & Cowburn, 1974; Barrantes et al., 1975; Damle & Karlin, 1978), and in solubilized AcChR [see review in Karlin et al. (1976)] and have helped to identify the polypeptide subunit carrying the recognition site for monoquaternary cholinergic ligands (Reiter et al., 1972; Karlin & Cowburn, 1973; Weill et al., 1974). Considerably detailed information on the surface topography of the recognition site and of some stereochemical aspects of agonist and antagonist binding has been gained from this approach.

Aside from the implications of specifically addressing the above-mentioned disulfide bond, it is known that the chemical modification of thiol groups in general brings about alterations in the physiological response of cholinergic excitable membranes. Reduction of the living electroplax with DTT, for example, inhibits the permeability response to acetylcholine (Karlin & Bartels, 1966). Subsequent alkylation of the reduced membranes renders the inhibition irreversible, while oxidation can restore the initial sensitivity to agonists. When the AcChR is integrated in its natural membrane environment, as opposed to in detergent micelles, the modification of thiol groups obviously involves sulfur-containing amino acid residues not only from the AcChR but also from other membrane constituents [see Karlin (1977)]. However, this does not detract from the possible implications of such chemical modification studies for understanding the properties of the AcChR itself. On the contrary, this description probably reflects more realistically the complex regulation of the redox balance in the living excitable membrane. In spite of the high density of the AcChR in this structure [see review in Barrantes (1979)], such balance is more likely to depend on multiple contributions from other constituents and to influence various receptor functional characteristics, among which one can specify the state of receptor aggregation (monomer–multimer equilibrium and receptor clustering), surface charge properties, and ligand binding phenomena. The present work concerns itself with the involvement of thiol groups in AcChR-rich membranes in the modulation of receptor affinities and cholinergic ligand recognition and with the peculiar effects of thiol modification on the action of partial agonists.

Material and Methods

Torpedo marmorata electroplax was dissected from freshly killed specimens obtained from the Biological Station of Arcachon, France, and kept in aquaria for a few days before processing. DTNB was purchased from Serva (Heidelberg). β-Mercaptoethanol, DTT, NEM, acetyl-, (acetylthio)-, car-

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[1] Abbreviations used: AcChR, acetylcholine receptor; BSA, bovine serum albumin; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; MPTA, [4-(*N*-maleimido)-phenyl]trimethylammonium; NEM, *N*-ethylmaleimide.

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bamoyl-, and succinylcholine, decamethonium, and *d*-tubocurarine were obtained from Sigma (Munich). α-Bungarotoxin was from Boehringer (Ingelheim), and its tritium derivative was purchased from Amersham Buchler (Braunschweig). α-Cobrotoxin was purified from the venom of *Naja naja atra* (obtained from K & K Laboratories) and radiolabeled as previously described (Barrantes, 1978). Suberyldicholine diiodide was a gift from Dr. J. Heesemann; (α-bromoacetyl)choline and MPTA were gifts from Dr. I. Silman (The Weizmann Institute).

Acetylcholine receptor rich membrane fragments were prepared from the electric organ of *T. marmorata* according to Cohen et al. (1972) or by a modification (to be published elsewhere) of the procedure of Sobel et al. (1977). This modified, shortened version of the purification scheme yielded maximal [^{3}H]-α-cobrotoxin binding activity in the membrane fraction sedimenting at the interface of 1.44–1.38 M sucrose. The membranes were stored in sucrose containing 0.1 M phenylmethanesulfonyl fluoride, 0.1 M EDTA, and 0.02% sodium azide at 4 °C and used within 4 days.

α-Toxin association kinetic measurements were performed as described previously (Barrantes, 1976, 1978) by using [^{3}H]-α-cobrotoxin (*Naja naja atra*, sp act. 2.9 Ci/mmol). The association rate measurements in the absence and presence of carbamoylcholine, with and without preincubation with the agonist prior to toxin addition [see Barrantes (1978)], were used to determine the affinity state of the membrane preparation as recommended by Quast et al. (1978).

Thiol Group Modification. Conditions for the oxidation, alkylation, and reduction of the AcChR-rich membrane fragments, as well as combinations of these thiol-directed reactions, are given in the text and figure legends.

Affinity Labeling. Concentrated stock suspensions of the AcChR-rich membrane fragments (specific activities typically in the order of 1 nmol of α-toxin sites per mg of protein) were diluted into 100 mM NaCl, 1 mM EDTA, and 1 mM Tris-HCl buffer, pH 8.0, containing 1 mM dithiothreitol to give concentrations of 250 μg of protein per mL. Reduction was conducted at room temperature for 30 min. The reaction was stopped by addition of 0.5 M sodium phosphate buffer, pH 6.7, and centrifugation of the samples at 4 °C in a Sorvall SS34 rotor at 20000 rpm for 40 min. The pellet was resuspended in 20 mM NaP_i buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA. Affinity alkylation with [4-(*N*-maleimido)phenyl]trimethylammonium was performed as reported by Barrantes et al. (1975) at a final concentration of 50 μM. After reacting for 5 min at 25 °C, the labeled membranes were washed by dilution and centrifugation at 20000 rpm for 40 min.

Fluorescence measurements were carried out on AcChR-rich membranes resuspended in the appropriate buffer systems (see figure legends), previously degassed and filtered through Millipore 0.45-μm filters, to give final protein concentrations of 30–100 μg/mL. The membrane suspensions (1.5 mL) were placed in 7 × 7 mm quartz cuvettes each fitted with a special injection–stirring device (Barrantes, 1976). Between 2 and 20 μL of ligand could be injected onto the mixing area above the cone of illumination within 2 to 3 s. All fluorescence experiments were carried out in the instrument designed by Rigler et al. (1974) with minor modifications as given in Barrantes (1978). The exciting light (200-W Xe–Hg arc) was passed through a 296.7-nm narrow band interference filter (Corion Corp.). Intrinsic fluorescence emission was collected above 320 nm (Schott WG-320 filter). The data were temporarily stored in Fabritek 1702 or Tracor Northern 1710 signal averagers. Analysis of the data was performed in a Digital PDP 11/20 minicomputer using an iterative nonlinear regression multiexponential fitting program written by L. Avery.

Assays. Protein concentrations were estimated by the method of Lowry et al. (1951) using BSA as a standard. The toxin binding activity of the membrane fragments was determined with the Millipore filtration technique (Olsen et al., 1972) using [^{3}H]-α-cobrotoxin or the DEAE-cellulose binding assay of Schmidt & Raftery (1973) and [^{3}H]-α-bungarotoxin in *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM NaP_i, pH 7) containing 0.1% BSA. Filters were previously soaked for 1 h in 1% BSA to reduce nonspecific binding of radiolabeled toxin (Barrantes, 1978). Sulfhydryl groups in the membrane fragments were determined by the method of Ellman as described by Chang & Bock (1977) using 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB, Ellman's reagent).

Results

Effects of Thiol Group Modification on Cholinergic Ligand-Mediated Inhibition of α-Toxin Association Kinetics. Measurements of the apparent rate of α-toxin association is an indirect but sensitive assay for the detection of ligand-mediated affinity changes in the AcChR. In the present series of experiments, the effect of various thiol reagents on the time course and extent of the ligand inhibition was followed in a wide range of concentrations on samples supplemented simultaneously with the colinergic effector and toxin ("no preincubation condition", $t \rightarrow 0$) and after prolonged incubation with the ligand prior to toxin addition ("maximal preincubation condition", $t \rightarrow \infty$).

As shown in Figures 1 and 2 and Table I, neither treatment with DTNB nor alkylation of the SH groups available in "native" membranes with NEM modified the extent of inhibition or the time course of onset of the affinity change exerted by typical mono- and bis-quaternary cholinergic agonists on the apparent rate of α-toxin association. The inhibitory effect displayed by nicotinic antagonists like *d*-tubocurarine or gallamine or by the mixed ligand decamethonium also remained unaltered after treatment with either DTNB or NEM. Only one affinity state could be detected with the rate measurements in the presence of antagonists; i.e., no state transitions occurred in the time range accessible to the assay with this type of ligand. No differences were apparent between membranes alkylated at the initial stages of preparation (homogenization) or after purification of the AcChR-rich fragments.

In contrast to the above lack of effects, the reduction of thiol groups with DTT produced marked alterations in the agonist response (Figures 1 and 2 and Table I). The degree of inhibition exerted by the agonist on toxin association kinetics decreased upon DTT reduction without alteration of the total number of toxin sites available at equilibrium or of the actual α-toxin association rate, as if (1) the chemical modification of the thiol groups almost completely hindered the agonist-mediated state transitions toward higher affinity forms and (2) the AcChRs were shifted, in the case of no preincubation conditions, to a state of even lower affinity than the native one (hereafter referred to as the "low_{DTT}" affinity state). On the contrary, the inhibition of α-toxin association kinetics produced by antagonists like gallamine, *d*-tubocurarine, or hexamethonium was not affected by reduction (Table I). Decamethonium was the only ligand tested for which reduction enhanced the degree of inhibition attained (Figure 1 and Table I).

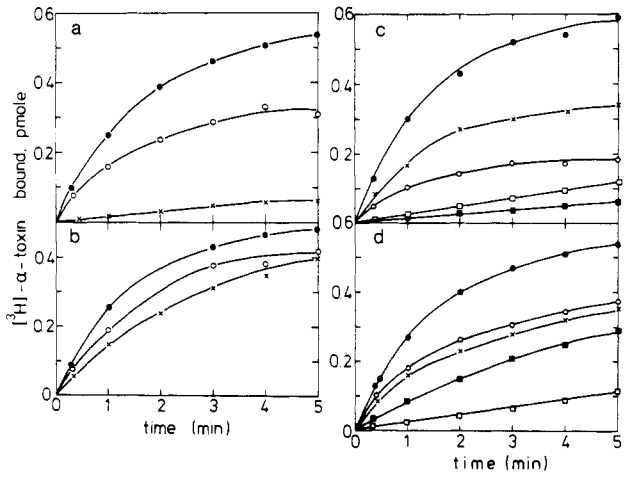

FIGURE 1: Examples of the kinetics of α-toxin binding to AcChR-rich membranes from *T. marmorata* as a function of time of exposure to the ligand and treatment with thiol reagents. (a) Carbamoylcholine. (●) Control kinetics in the absence of agonist. No differences were observed in the α-toxin–AcChR complex formation (k_{ass} = (1.2 ± 0.3) × 10^7 M^{-1} min^{-1}) in the absence or presence of 1 mM DTNB or 5 mM NEM. (○) Same as in (a) in the presence of 10 μM carbamoylcholine on normal or 1 mM DTNB (30 min) treated membranes. Agonist and toxin were added simultaneously to the membrane suspension ($t \rightarrow 0$ conditions). (×) Toxin binding kinetics measured after 30-min ($t \rightarrow \infty$ conditions) preincubation with 10 μM carbamoylcholine, either on normal or DTNB-treated membranes. (b) The rate measurements shown in (a) are repeated on membranes pretreated with 5 mM DTT + 1 mM NEM (30 min each) prior to the addition of 100 μM carbamoylcholine and/or toxin addition. (●) Control curve, as in (a); (○) $t \rightarrow 0$ conditions; (×) $t \rightarrow \infty$ conditions as in (a). (c) Effect of DTT reduction on decamethonium-induced transitions. (●) Control kinetics, both for normal and 5 mM DTT reduced membranes; (×) $t \rightarrow 0$ conditions, normal membranes in the presence of 80 μM decamethonium; (□) $t \rightarrow \infty$ conditions, normal membranes; (○) after 30-min exposure to 5 mM DTT; (■) $t \rightarrow \infty$ conditions with decamethonium after 30-min reduction with 5 mM DTT. (d) Effects of reduction + alkylation on decamethonium-induced transitions. Symbols are as in (c). The DTT reduction as above was followed by alkylation with 5 mM NEM for 30 min prior to the kinetic measurements. Initial receptor and toxin concentrations were 5 and 1.5 nM in all cases; temperature was 20 °C.

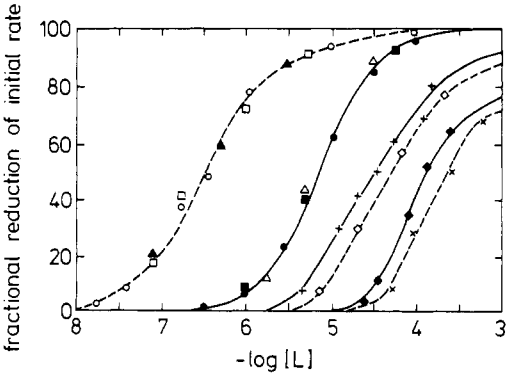

FIGURE 2: Concentration dependence of the carbamoylcholine-mediated inhibition of the apparent initial rate of [^{3}H]-α-cobrotoxin association to normal and thiol-modified AcChR-rich membranes. Nonpreincubation conditions ($t \rightarrow 0$) for normal (●), DTNB-treated (Δ), or NEM-treated (■) membranes and, correspondingly, 30-min preincubation conditions ($t \rightarrow \infty$) for normal (○), DTNB-modified (▲), and NEM-modified (□) membranes. Lower affinity forms result from DTT treatment, both for $t \rightarrow 0$ (□) and $t \rightarrow \infty$ (+) conditions. The ultralow-affinity states for carbamoylcholine after DTT + NEM combined treatment are shown for the $t \rightarrow 0$ conditions (×) and for maximal preincubation conditions (■). Data were taken from experiments like the one shown in Figure 1.

The effects of DTT reduction followed by NEM alkylation were studied in another series of experiments. The same qualitative results observed upon DTT reduction were also

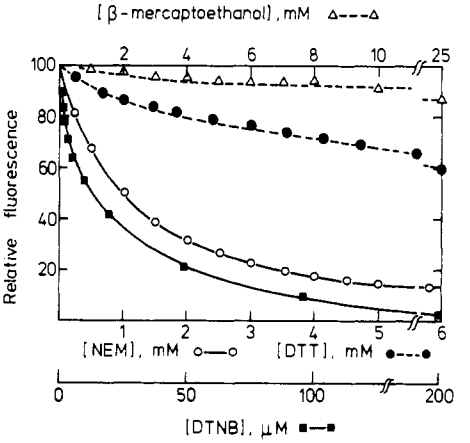

FIGURE 3: Titrations of the intrinsic fluorescence signal arising from AcChR-rich membranes with thiol reagents. Stock concentrated suspensions of membranes (2–5 mg/mL) were diluted in 1.5 mL of 20 mM NaP_i buffer, pH 7.4, and allowed to equilibrate for 30 min at 20 °C with intermittent stirring. The final protein concentration was 80 μg/mL, and the AcChR concentration was 75 nM in terms of [^{3}H]-α-cobrotoxin binding sites. Concentrated stock solutions of the thiol reagents were added in small aliquots (2–5 μL) and allowed to mix with continuous magnetic stirring for 5 min before the fluorescence measurements were taken. The readings were integrated for 15-s intervals. Other experimental conditions are given under Material and Methods. Notice the difference in reagent concentrations needed to accomplish similar degrees of intrinsic fluorescence quenching with DTNB (■), β-mercaptoethanol (Δ), NEM (○), and DTT (●).

apparent after alkylation of the thiols made available by reduction, including the ability of agonists to induce affinity state transitions (Figure 1 and Table I). Quantitatively, however, the degree of inhibition exerted by agonists on DTT + NEM treated membranes was substantially lower than either that observed in normal or in DTT-reduced membranes. Apparent $[L]_{0.5}$ values for carbamoylcholine inhibition under these conditions were two- to sixfold lower than in controls (Figure 2 and Table I). The effect of decamethonium on DTT + NEM treated membranes followed the pattern observed with the typical agonists tested. As shown in Figure 1d, the potency of decamethonium as an inhibitor of toxin rates diminished after the treatment. The effect is particularly noticeable when compared to the action of the same ligand on membranes which were only reduced (Figure 1c).

The essential reversibility of the effect of reduction was attested in another series of experiments, where removal of DTT by dilution and centrifugation and subsequent reoxidation with DTNB was performed. Concentrations of 200–400 μM DTNB were needed to accomplish the reoxidation of the membranes. Reoxidized membranes displayed the time and concentration dependence of the agonist-mediated inhibition observed in the original control samples. That is, the effect of DTT reduction was found to be completely reversible. On the other hand, DTT-reduced + NEM-alkylated membranes were not capable of regaining agonist sensitivity (and by inference, their low-affinity form) after reoxidation, in contrast to the effect of DTT reduction or NEM alkylation alone (see above).

Changes in Intrinsic Fluorescence in AcChR-Rich Membranes upon Thiol Modification. Treatment of the AcChR membranes with DTNB was by far the most effective reaction affecting the intrinsic fluorescence via thiol modification. Practically complete abolition of the fluorescence emission occurred at ~200 μM DTNB (Figure 3). When the changes of transmission at 412 nm were followed in control experiments to assess the amount of thiol groups using the classical Ellman's

Table I: Summary Effects of Cholinergic Ligands on Normal and Thiol-Modified AcChR Membranes Assessed by the Apparent Rate of α-Toxin Association and the Intrinsic Fluorescence Response

ligand	conditions[a]	$[L]_{0.5}$[b]	postulated affinity state[c]	intrinsic fluorescence response[d]
agonists				
carbamoylcholine	control ($t \rightarrow 0$), DTNB, NEM	20 μM	low	quenching
	control ($t \rightarrow \infty$), DTNB, NEM	0.5 μM	high	
	DTT ($t \rightarrow 0$)	70 μM	low_{DTT}	fast quenching, slow increase
	DTT ($t \rightarrow \infty$)	50 μM	$high_{DTT}$	
	DTT + NEM ($t \rightarrow 0$)	200–350 μM	ultralow	
	DTT + NEM ($t \rightarrow \infty$)	100 μM	$low_{DTT+NEM}$	
suberyldicholine	control ($t \rightarrow 0$), DTNB, NEM	0.5 μM	low	quenching
	control ($t \rightarrow \infty$), DTNB, NEM	10 ± 2 nM	high	
	DTT + MPTA			fast quenching, slow increase
(α-bromoacetyl)choline	control ($t \rightarrow 0$)	0.5–1 μM	low	quenching
	control ($t \rightarrow \infty$)	5–10 nM	high	
	DTT ($t \rightarrow 0$)			fast quenching, slow increase
(acetylthio)choline	control ($t \rightarrow 0$)	ND[e]		quenching
antagonists				
flaxedil (gallamine)	control ($t \rightarrow 0$, $t \rightarrow \infty$), DTNB, NEM, DTT or DTT + NEM	1–2 μM	high?	no detectable effect
d-tubocurarine	same as above	0.5 μM	high?	no detectable effect at low concns (<50 μM)
hexamethonium	same as above	20 μM	high?	same as above
partial agonists				
succinylcholine	DTT	ND		increase
	control ($t \rightarrow 0$)	ND		quenching
decamethonium	control ($t \rightarrow 0$), DTNB, NEM	50 μM	high	quenching
	control ($t \rightarrow \infty$), DTNB, NEM	0.7 μM	low	
	DTT	higher than in the controls		fluorescence increase
	DTT + NEM	lower than in the controls		

[a] Control refers to untreated (native) membranes. The time regimes explored, $t \rightarrow 0$ and $t \rightarrow \infty$, correspond to the simultaneous ligand and toxin addition and to the 30-min preincubation with the ligand, respectively. Equilibration for 30 min sufficed for achieving equilibration of the ligand-induced affinity conversion. Intermediate times of ligand preincubation, explored for some agonists, are omitted for the sake of clarity. DTNB, NEM, DTT, and DTT + NEM refer to the corresponding treatment with these reagents as indicated in the figure legends and under Material and Methods. [b] Determined from the apparent rate of [^{3}H]-α-cobrotoxin (*Naja naja atra*)-AcChR complex formation as shown in Figures 1 and 2. [c] The nomenclature is maintained in spite of the present findings to facilitate comparison with literature values. It is clear, however, that at least the low-affinity forms should be considered provisional. [d] Conditions for the listed qualitative fluorescence changes in the time scale of seconds to minutes and for the thiol modifications, as in Figures 5–7. [e] ND = not determined.

reaction in intact membranes or after sodium dodecyl sulfate solubilization, higher concentrations of DTNB were needed to titrate these groups after solubilization. A total of 230 ± 50 nmol of SH groups per mg of protein was found in denatured membranes and about two-thirds this amount in intact membranes (160 ± 30 nmol/mg of protein). Given the average specific activity of the membrane preparations, this corresponds roughly to 20–25 SH residues/AcChR monomer of M_r ~250 000 (Reynolds & Karlin, 1978), a value in close agreement with that of Eldefrawi et al. (1975). Taking into account the results obtained in other membranous systems with DTNB and the low partition coefficient of this reagent in 1-octanol–water (Murphy, 1976), it is likely that the Ellman's reagent detected only accessible free SH groups in intact membranes and not the total thiol content made apparent upon $NaDodSO_4$ solubilization. It is also worth noticing that the concentrations of DTNB needed to achieve maximal fluorescence quenching (Figure 3) coincide with those reported recently by Hamilton et al. (1979) for optimally converting the AcChR monomer to higher oligomeric forms. Reduction of the AcChR membranes with DTT or β-mercaptoethanol also resulted in diminution of the intrinsic fluorescence, but maximal values of 40 and 10% quenching were obtained with each reagent respectively at higher concentrations than those required with DTNB (Figure 3). Alkylation with NEM, without prior reduction of the membranes, produced quenching levels in between those of the oxidizing and reducing agents (Figure 3).

The kinetics of the protein fluorescence changes induced by thiol group modification was followed in the time range of seconds to minutes. Within experimental sensitivity, all thiol reagents produced the same qualitative pattern, composed of two distinct phases. This is exemplified by the kinetics of the DTNB reaction, as shown in Figure 4. A rapid phase consisting of the quenching of the intrinsic fluorescence was apparent upon injection of the reagent. Given the dead time of injection + mixing, the initial 2 to 3 s of this rapid phase is beyond the resolution of the present technique (Barrantes, 1976). The subsequent slower phase was of opposite sign and partially reversed the initial fluorescence quenching within seconds. The slow phase followed approximately the time course of DTNB consumption at the given reactant concentrations and temperature, being hence equivalent to the development of the 2-nitro-5-thiobenzoate anion and Ellman's reaction adducts.

Although DTT had on its own a moderate quenching effect on native membranes, the quenching elicited by NEM could be partially reversed by DTT treatment of NEM-alkylated membranes. After reaching maximal quenching with NEM (80–85%, 5 mM reagent, Figure 3), up to 70% of the intrinsic fluorescence could be restored to its initial level by DTT reduction. Given the chemical nature of the NEM alkylation, it is reasonable to infer that the restoration of the fluorescence signal under these circumstances must necessarily involve thiol groups other than those affected by NEM in intact membranes, presumably by breakage of disulfide bonds not accessible to NEM and/or formation of mixed disulfides with free SH groups.

Pretreatment of the AcChR membranes with cholinergic ligands did not significantly affect the *overall* effects of re-

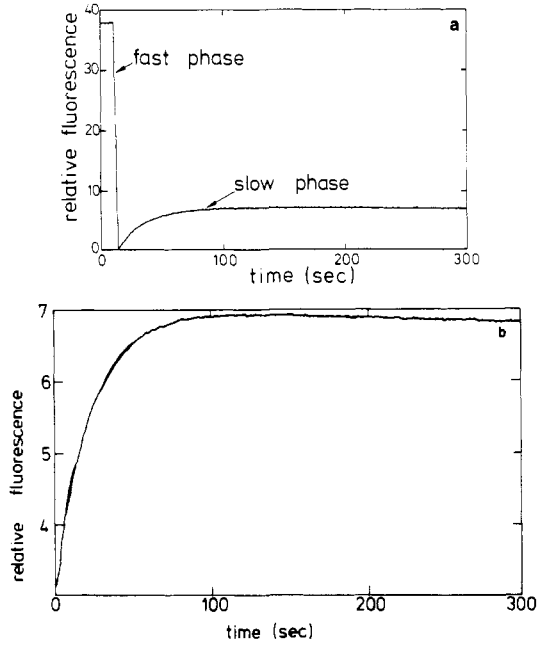

FIGURE 4: Kinetics of the DTNB effect on the intrinsic fluorescence of the AcChR-rich membranes. The membrane suspension (70 μg of protein per mL, 65 nM in α-toxin sites) was equilibrated for 30 min at 20 °C in 20 mM NaP_i buffer, pH 7.4. Injection and mixing of DTNB were accomplished within less than 4 s, and the dilution effect, determined in control experiments, amounted to 0.15% of the fluorescence signal. (a) shows the complete course of the DTNB effect at a final concentration of 40 μM, consisting of a rapid quenching phase followed by a slower partial reversal. (b) The slow phase followed a single exponential time course, as determined by multiexponential fitting analysis of the experimental points with a theoretical curve corresponding to a half-time of 22 ± 1 s. The base line drift was accounted for by a sloping line of 0.06 s^{-1}. [Its occurrence is discussed in Barrantes (1978).] Other kinetically distinguishable sets of sulfhydryls could be detected at higher DTNB concentrations or at different temperatures.

duction or alkylation on the intrinsic fluorescence. A similar lack of effectiveness in protecting from NEM alkylation is observed with cholinergic ligands in the case of the muscarinic receptor from smooth intestinal muscle (Stubbins & Hudgins, 1971). DTT reduction of the nicotinic AcChR is also not affected by *d*-tubocurarine (Rang & Ritter, 1971). High concentrations of suberyldicholine had a small but appreciable effect on the DTNB oxidation of the membranes; preincubation with 20 μM agonist reduced by ~5% the amplitude of the DTNB quenching and accelerated the rate of the slower phase (half-times 23 and 13 s in control and agonist-treated samples, respectively; 40 μM DTNB). Similar qualitative changes were observed in AcChR membranes incubated with saturating amounts of α-bungarotoxin; the rate of the slower phase reached in this case half-times of ~7 s. Cholinergic ligands may therefore hinder certain thiol groups from reacting with the oxidizing agent. Alternatively, it could be argued that the receptor states stabilized at high ligand concentrations differ in the structural arrangement of their thiol groups from the native AcChR. An acceleration of the collisional quenching constant and changes in the average static and dynamic availability of fluorophores in the AcChR were observed by using acrylamide and lipid nitroxide quenching in the presence of suberyldicholine (Barrantes, 1978).

Effects of Thiol Group Modification on the Fluorescence Response to Cholinergic Ligands: Altered AcChR Physical States. The action of cholinergic agonists on the intrinsic fluorescence of AcChR membranes consists of a time- and concentration-dependent quenching, as previously reported for

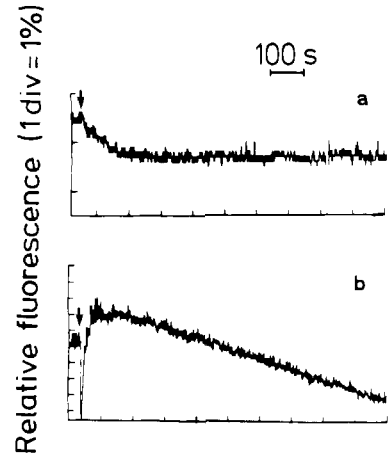

FIGURE 5: Lack of effects of NEM alkylation on the intrinsic fluorescence response of the AcChR membranes to carbamoylcholine and its alteration upon DTT reduction. In (a) 5 mM NEM alkylated membranes, at an initial concentration of 50 μg of protein per mL (45 nM in α-toxin sites), were supplemented at the point indicated by the arrow with a concentrated solution of carbamoylcholine (5 μL of ligand sandwiched in between 5-μL bottom and 5-μL upper cushions of buffer) to give a final concentration of 60 μM carbamoylcholine. No differences were observed between the responses obtained in control [see Bonner et al. (1976)] and NEM-alkylated membranes. The dilution effect amounted to 0.3%. (b) In marked contrast to the previous results, the response to 60 μM carbamoylcholine in DTT + NEM treated membranes consisted of an accelerated initial quenching of the intrinsic fluorescence, amounting to ~4.5%, followed by a slower phase of opposite sign, amounting to an increase in relative fluorescence of ~6%; thereafter the signal decreased at a steady rate. Reduction was accomplished with 5 mM DTT for 30 min, followed by 5 mM NEM alkylation for 30 additional min. Excess reagents were eliminated by centrifugation prior to the fluorescence experiments which were conducted in 10 mM NaP_i buffer containing 100 mM NaCl, pH 7.4, at 20 °C.

acetylcholine, carbamoylcholine, and suberyldicholine (Bonner et al., 1976; Barrantes, 1976, 1978). The same effect was observed in the present experiments with (acetylthio)-, succinyl-, and (α-bromoacetyl)choline and decamethonium (Table I). In agreement with the results from the toxin kinetic experiments, DTNB-modified membranes or membranes alkylated with NEM without prior reduction did not differ from the controls in response to ligands (Figure 5a and Table I). Reduction with DTT or reduction followed by NEM alkylation, on the other hand, drastically altered the fluorescence responses. The agonist-induced quenching had a faster onset and larger amplitude but spontaneously *reverted* to fluorescence levels higher than those observed before agonist addition (Figure 5b).

Two bis-quaternary ammonium compounds produced a qualitatively different effect on DTT-reduced membranes. Addition of succinylcholine or decamethonium to these membranes was not accompanied by a quenching (within the experimental resolution of the technique) but by an *increase* of fluorescence (Figure 6). This inversion of the fluorescence response after DTT treatment was not observed with antagonists. For instance, hexamethonium, a mild antagonist, normally reverses the quenching effect of agonists and produces a negligible increase of fluorescence in normal membranes at relatively high concentrations. When its effect was assayed in DTT-treated membranes, the overall effect of this ligand was to leave the intrinsic fluorescence unaltered (Figure 6). Fast kinetic studies will be necessary to resolve the effects of these agents in normal and thiol-modified membranes.

Although three types of sulfhydryls are available in the presence of DTT (the cleaved disulfide in the vicinity of the AcChR recognition site, other SH groups in the receptor and

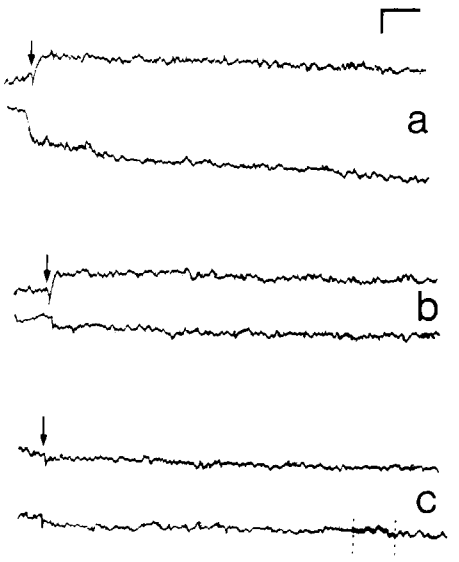

FIGURE 6: Alteration of normal responses to bis-quaternary cholinergic effectors after reduction and lack of effects on fluorescence response to hexamethonium. In all cases the top trace corresponds to the 5 mM DTT reduced sample and the bottom one to the control. (a) Fluorescence response to the addition of succinylcholine chloride (final concentration of 100 μM) to AcChR membranes (1.1 nmol of [^{3}H]-α-toxin sites per mg of protein, 90 μg/mL). Other experimental conditions are as in Figure 5. Note the diametrically opposite effects of the agonist on normal membranes, 2% relative quenching, and on reduced samples, in this case a 1.8% fluorescence *increase* (after accounting for the dilution effect). (b) Inversion of the effect of 100 μM decamethonium. (c) 100 μM hexamethonium produces no net change in the intrinsic fluorescence when applied to DTT-reduced membranes or to untreated control samples. All records were obtained in a Tracor 1710 signal averager at a rate of 1 point/s. Ligands were injected at the point indicated by the arrows. The horizontal mark (top right corner) and the intensified region in the bottom curve c correspond to 50 s; the vertical mark (top right corner) indicates 1% relative fluorescence intensity.

other proteins, and those of DTT itself), conditions can be met where active-site-directed probes can be reacted almost exclusively with the SH groups generated at the AcChR binding site (see beginning of article). The experiments which follow were conducted with the aim of establishing with greater certainty whether the particular S–S bond in close proximity to the recognition site was affected by the DTT reduction and whether such chemical modification could, in turn, be responsible for the alterations in ligand discrimination.

[4-(*N*-Maleimido)phenyl]trimethylammonium (MPTA) behaves as a mild competitive antagonist of the normal AcChR and fixes the receptor in an inactive conformation when following reduction (Karlin & Winnik, 1968). We have previously used this affinity reagent to label half of the toxin binding sites in *T. marmorata* membrane fragments (Barrantes et al., 1975). Similar experimental conditions have been used in the present work to study the effects of affinity alkylation on the AcChR fluorescence response to ligands. Figure 7 shows the contrasting effects of the powerful agonist suberyldicholine on the intrinsic fluorescence of normal and DTT-reduced, MPTA-alkylated AcChR membranes. The normal response consists of the intrinsic fluorescence quenching from the resting value F_0 to a new level, F_1. The ligand concentration was deliberately chosen in the example shown because the kinetics of the transition between these two levels is faster than the time resolution of the technique, and thus any departure from this pattern becomes readily apparent. This is precisely the case in the thiol-modified membranes; an initial rapid quenching phase follows the agonist addition ($F_2 \rightarrow F_3$). The amplitude of this phase is larger than that observed in normal

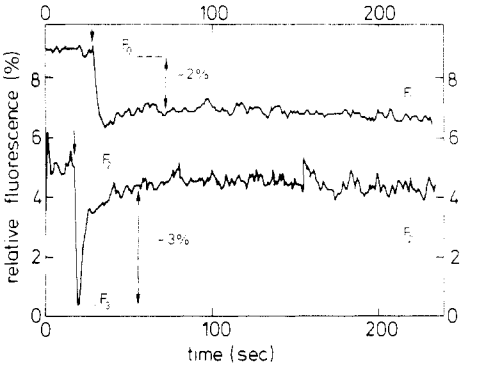

FIGURE 7: Effect of affinity alkylation on agonist-mediated fluorescence response. Upper trace: control. Suberyldicholine (20 μM) was added to the membrane fragments (75 μg of protein per mL; 70 nM in α-toxin sites) at the point indicated by the arrow. Membranes were resuspended after centrifugation (to match the conditions of the affinity labeling experiment) in 20 mM NaP_i buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 0.1 M sucrose. Lower trace: suberyldicholine (20 μM) effect on 0.2 mM DTT reduced, 50 μM MPTA alkylated membranes. The quenching effect ($F_2 \rightarrow F_3$) is larger than in the controls, but it rapidly reverses and gives rise to an increase in fluorescence (half-time 8.7 s) which reverts the signal to the original level ($F_{2'}$) (after accounting for the dilution).

membranes. A slower change of opposite sign ($F_3 \rightarrow F_{2'}$) supervenes, reverting the fluorescence level to that observed before addition of the agonist. The final state reached differed from the initial (F_0) and final (F_1) levels obtained without chemical modification of the thiol groups. The kinetics of the slower phase followed an exponential time course with half-times of a few seconds, significantly slower than those of the rapid quenching phase. Although MPTA or (α-bromoacetyl)choline reacts with half of the toxin sites [see also Damle & Karlin (1978)], preincubation with the toxin abolished more the 95% of the reaction with either of these two affinity reagents. This observation speaks for the specificity of the labeling.

Discussion

Alteration of Affinity States by Thiol Group Modification. Exposure of the membrane-bound AcChR to certain cholinergic ligands, most notably with typical agonists of the nicotinic system, is known to cause a concentration- and time-dependent increase in the affinity of the receptor for such ligands (Weber et al., 1975; Barrantes, 1976, 1978; Colquhoun & Rang, 1976; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978; Lukas et al., 1979; Moore & Raftery, 1979). In the present case treatment of the native AcChR membranes with DTNB or alkylation with NEM did not alter this property. Furthermore, typical antagonists did not appreciably induce affinity changes as judged by the toxin rate parameter, nor were such changes influenced by any of the thiol modification procedures. The lack of effects of alkylation agrees with the observations of O'Brien & Gibson (1977) and Chang & Bock (1977) on the acetylcholine binding properties of alkylated, detergent-solubilized AcChR.

DTT-reduced or DTT + NEM treated membranes, on the other hand, showed a marked diminution of their responses to agonist-mediated induction of higher affinity forms. Quantitatively, the effect obtained by reduction alone was less pronounced than that observed after alkylation of the reduced thiols (Table I). However, this may only be an apparent difference due to partial reoxidation of the samples in the course of the assay. These observations show concurrence with those of Karlin & Bartels (1966), who found that *p*-(chloromercuri)benzoate inhibited agonist-mediated permeability in

the electroplax, whereas NEM by itself did not. The changes mediated by reduction on agonist affinities are also in agreement with those reported by Schiebler et al. (1977) on acetylcholine-mediated ^{22}Na efflux from *Torpedo* microsacs.

Could the modulation of affinity states resulting from thiol modification bear relevance to the function of the AcChR in situ? Recent electrophysiological experiments in which dose–response curves were obtained under rigorously controlled ionophoresis in voltage-clamped endplates yielded apparent dissociation constants for carbamoylcholine of 300–400 μM (Dreyer et al., 1978; Dionne et al., 1978), i.e., within the range of the ultralow affinity state for this agonist in vitro (Table I). The striking similarity between the values, minor differences aside,[2] raises the possibility that in the living cell the metabolic machinery maintains a (dynamic) redox balance normally favoring affinity states similar to those statically captured in vitro upon man-tailored chemical modification of thiol groups. The reasons for favoring such affinity states are as yet unknown, but one could conceive of such reasons as being related to the optimization of the gating function by the AcChR-controlled channel. Fast and repetitive channel gating could, in the nicotinic type of synapse, be advantageously associated with a short residence time of the neurotransmitter on the AcChR in the liganded or occupied state (closed–open). If the binding step(s) is rate limiting, it is effectively the rapid dissociation of the ligand from a low-affinity AcChR form that would provide the final termination of gating activity.[3]

State Transitions and Thiol Group Modification. Not only are the affinity *states* detected in the presence of agonist the same in native, DTNB-modified, and NEM-alkylated membranes but also the capacity to undergo state *transitions* is qualitatively and quantitatively preserved in these three cases. This property is also present, though quantitatively diminished, in reduced or reduced + alkylated membranes. Furthermore, the transient nature of the low_{DTT}-affinity state is evidenced by the restoration of native agonist sensitivity, in terms of affinity shifts, upon reoxidation, a finding in agreement with that of Moore & Raftery (1979). These authors accomplished the reversal by prolonged spontaneous reoxidation of the samples. In contrast, reduced + alkylated membranes apparently lose the ability to regain the native affinity state, although they show agonist-induced transitions.

The recent application of fast kinetic techniques to the study of AcChR-ligand interactions in vitro [see review in Barrantes (1979)] extended the original observations on AcChR state transitions obtained by indirect methods and provided quantitative estimates of the rates of such transitions. The kinetic and equilibrium parameters derived therefrom provided evidence for the existence of a nonnegligible proportion of a high-affinity ("desensitized") conformation of the AcChR in addition to the predominant low-affinity ("resting") conformer (Barrantes, 1978). This finding has been confirmed with extrinsic fluorescence spectroscopy (Heidmann & Changeux, 1979) and more recently with fast filtration techniques (Cohen & Boyd, 1979) and is in accordance with the original postulation of Katz & Thesleff (1957) in relation to the occurrence of these states (preexisting ligand binding) in vivo. In the present case, the multiplicity of AcChR states in normal and thiol-modified membranes precludes any elaboration of the data in terms of detailed kinetics. The present observations clearly indicate in a qualitative manner, however, that the normal reaction pathways are not followed in reduced or reduced + alkylated membranes. The alteration of agonist reaction rates in modified membranes revealed by intrinsic fluorescence spectroscopy together with the observation of modified state transitions normally occurring in the time course of seconds suggests that the process ascribed in vitro to the desensitization phenomenon is also influenced by chemical modification of the thiol groups.

Alteration of the AcChR Recognition Ability. In addition to the above alterations of affinity states of the AcChR and transitions thereof, it appears that the reductive cleavage of certain disulfide bonds in the receptor results in specific changes of its recognition capacity toward cholinergic ligands. This is exemplified by the "antagonist-like" response to a typical agonist like carbamoylcholine after DTT reduction + NEM alkylation (Figure 5) or to suberyldicholine after affinity alkylation (Figure 7), as well as the effects of partial agonists after reduction (Figure 6). The results from the affinity alkylation experiments strengthen the view that the active-site disulfide group is a primary, if not a preferred, target of the reductive attack and that this group is essential for the correct discrimination of the ligand. In the case of affinity acylation with (α-bromoacetyl)choline, which reacts with half of the α-toxin sites of the AcChR (Damle et al., 1978) as MPTA does (Barrantes et al., 1975), the modified receptor is still able to bind the agonist after treatment with antagonists, as is observed in the living electroplax (Silman & Karlin, 1969). The agonist response under these conditions is, however, abnormal (F. J. Barrantes, unpublished results). These results could be taken as indirect though strong evidence for the existence of nonequivalent ligand recognition sites, in agreement with the interpretation of Damle & Karlin (1978). The question of whether one disulfide bridge is absent in one of the recognition sites or of its decreased susceptibility to reduction cannot be answered from the available information. Irrespective of this presumptive half-of-the-sites reactivity, the disruption of the disulfide-bonded structure of the AcChR is a key factor in cholinergic ligand discrimination.

A Hypothesis on the Modulation of Partial Agonism by the Redox State in the Membrane. At variance with the results from the oxidation or initial alkylation experiments, the decamethonium-induced depression of toxin apparent rate was enhanced in reduced membranes, an effect diametrically opposite to that observed with the other agonists. Also, the intrinsic fluorescence response of the AcChR membranes to this ligand was reversed after DTT reduction. Other peculiar pharmacological effects are also apparent with decamethonium: the agonist action in the living electroplax of *Electrophorus* (Changeux & Podleski, 1968) or *Torpedo* (Moreau & Changeux, 1976) contrasting with the antagonist-like properties observed in vitro with membranes from the same source (Sugiyama et al., 1976) and the potentiation effects of reduction on the decamethonium-induced depolarization in the electroplax (Podleski et al., 1969; Karlin, 1969) and on the binding properties of the purified AcChR from *Electrophorus* (Meunier et al., 1974). Can all these effects be rationalized on a common basis? The hypothesis can be

[2] The main source of uncertainties in the two sets of values probably lies in the time limitations of the toxin binding assay. Discrepancies in the absolute values of $[L]_{0.5}$ most likely result from the complex combination of several experimental parameters (sampling techniques, ionic composition of the assay media, type of toxin, etc.), the method of analysis of the data, and the theoretical models proposed to account for the observed toxin kinetics [compare, for example, Weiland et al. (1977), Quast et al. (1978), Weiland & Taylor (1979), and Lukas et al. (1979)].

[3] After submission of this manuscript a paper appeared (Nelson & Sachs, 1979) showing the occurrence of multiple channel gating events in developing muscle, a finding which lends additional support to the above hypothesis. For a theoretical treatment of the problem, see Colquhoun & Hawkes (1977).

formulated that the contrasting effects of this "partial agonist" (and presumably of other ligands displaying local anesthetic-like blocking effects and some full agonists) are intimately related to and *modulated* by the redox state of the excitable membrane. The changes in activity observed with hexamethonium after reduction in the eel electroplax (Karlin & Winnik, 1968) or in the chick biventer muscle (Rang & Ritter, 1971) could also fall into this category.

The voltage sensitivity of the blocking effect produced by decamethonium (Adams & Sakmann, 1978) and other noncompetitive antagonists (and more recently also by the formerly pure competitive antagonists) has lent support to the notion that the binding site mediating this effect is located at some depth in the membrane, in a region sensitive to the electric field (Kordaš, 1970). The inferred blocking of open AcChR-controlled channels which stemmed from the above notion has developed into a widely accepted mechanism of noncompetitive antagonism (Adams, 1976). Formally, however, the same effects can be accounted for by allosteric mechanisms in which the blocker affects primarily a heterotopic local anesthetic site and, indirectly, the recognition unit of the AcChR. Channel occlusion would result in this case from conformational transitions in the recognition unit (including both homo- and heterotopic sites), propagated into the gating unit without involving plugging of the channel by the ligand itself. On these lines the marked increase in affinity of the AcChR for decamethonium after reduction and the transformation of the agonist responses into antagonist-like responses to this ligand after reductive cleavage (1) are reminiscent of the synergic effects of local anesthetics on the desensitization phenomenon in vivo and on the low- to high-affinity conversion in vitro and (2) make apparent the crucial role of disulfide bond cleavage in bringing forth, or maximizing, the noncompetitive part of decamethonium activity. The simplicity of the "open-channel blocking" hypothesis is, as Neher & Steinbach (1978) put it, appealing. It would thus appear appropriate to study in detail the effect of thiol group modification in relation to the action of local anesthetics and other noncompetitive blockers. Studies of this type may not only contribute to our understanding of the participation of these groups in AcChR function in general (aggregation and clustering of receptors, ontogenetic stabilization of the synapse, etc.) but may also provide a clue to elucidating the nature of the coupling between the entities carrying the recognition and gating functions of the AcChR.

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Modulation of Transcription from Chromatin Assembled in Vitro†

Lenē J. Holland* and Brian J. McCarthy

ABSTRACT: A small plasmid DNA was assembled into chromatin in vitro by incubation in an extract prepared from eggs of *Xenopus laevis*. The plasmid DNA contained the regulatory region of the *Escherichia coli lac* operon, the transcription of which is under positive regulation by catabolite activator protein (CAP) and negative regulation by *lac* repressor. After incubation in the egg extract the plasmid DNA acquired ~60% of the predicted maximum number of nucleosomes. Chromatin was treated with protein and DNA cross-linking agents prior to transcription in order to demonstrate that regions of the DNA organized into nucleosomes served as templates for transcription. Cross-linking abolished transcription of chromatin but had no effect on transcription of DNA, suggesting that transcription of untreated chromatin was not solely attributable to nucleosome-free regions. In support of this conclusion, the average size of the RNA transcribed from chromatin was ~1000 bases, which was ~5 times longer than the average distance between nucleosomes. Transcription of in vitro assembled plasmid chromatin by *E. coli* RNA polymerase was stimulated by catabolite activator protein. The CAP-mediated stimulation of transcription was detectable as an increase in total transcription that was specific to chromatin made from a plasmid containing the *lac* regulatory DNA sequences. The specific increase in the amount of RNA whose synthesis was initiated within the *lac* region was demonstrated by hybridization of transcription products to complementary DNA fragments bound to nitrocellulose filters. Preliminary investigation of the action of *lac* repressor suggested that it also modulated transcription from the chromatin template.

The DNA in both transcribed and untranscribed regions of eucaryotic chromosomes is associated with histones to form nucleosomes [Lacy & Axel, 1975; for a review, see Chambon (1978)]. However, it is clear from the appearance in the electron microscope and from sensitivity to nuclease digestion that transcriptionally active chromatin differs in organization from inactive chromatin (Foe, 1978; McKnight et al., 1978; Weintraub & Groudine, 1976; Garel & Axel, 1976; Bonner et al., 1974; Gottesfeld & Partington, 1977). In order to examine how transcription occurs through nucleosomes and what structural alterations underly changes in template activity, efforts have been directed toward the development of in vitro transcription systems (Williamson & Felsenfeld, 1978; Wasylyk et al., 1979b; Ng et al., 1979).

In this paper we describe an in vitro transcription system in which the template was chromatin that had been assembled in vitro by incubating DNA in a crude extract prepared from the eggs of the toad *Xenopus laevis*. Laskey et al. (1977) showed that this extract contains the histones and all other factors required for the assembly of DNA into nucleosomes. It seems likely that chromatin is assembled in the egg extract under conditions similar to those for synthesis of chromatin in vivo.

The DNA used for the assembly into chromatin was a bacterial plasmid containing a segment of DNA with the regulatory region of the *Escherichia coli lac* operon (Miller

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