Site-directed disulfide reduction using an affinity reagent: application on the nicotinic acetylcholine receptor

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Abstract The aim of this study was to present a new concept of site-directed reduction of disulfide bonds based upon the use of an affinity ligand harbouring a readily oxidizable dithiol. The cysteine bond involved in the acetylcholine binding site of the AChoR was specifically reduced by a carbamylcholine analogue. The ligand, in its oxidized form, was characterized by an affinity constant of 20 μM for the agonist binding site. In its dithiol form, it specifically reduced the disulfide between Cys-192 and Cys-193 on the α-subunits of the nicotinic acetylcholine receptor. This reduction needed 10 times lower concentration when carried out with site-directed reducing agent (ARA) than with DTT, and was highly specific for the α-subunits. The contribution of the carbamylcholine moiety of the site-directed reducing agent was clearly demonstrated in kinetic studies where reduction abilities of ARA, DTT and the methylated analogue of ARA (MeRA) were compared. At the same concentration (20 μM), DTT and MeRA had a 25 times lower initial rate of reduction than ARA. With 200 μM of DTT this initial reduction was still 4 times lower. Furthermore, the use of a maleimido undecagold cluster which specifically labeled the reduced nicotinic receptor opens the way to structural analysis of the agonist binding site by electron microscopy. These results demonstrate the potency of this kind of site-directed reducing agent for structural study of receptors or enzymes involving a disulfide bond in their active site.

Key words: Site-directed disulfide reduction; Nicotinic acetylcholine receptor

1. Introduction

Disulfide bonds are known to be essential elements of protein structure. They can be easily reduced (e.g. with DTT, β-mercaptoethanol, $NaBH_4$) and the cysteines can be specifically labeled (e.g. with iodoacetic acid, NEM). When a disulfide bond is located near a protein active site, its cysteines can be specifically labeled by an affinity probe after DTT reduction. However, in such an approach, some other disulfide bonds may also be reduced. We propose here a new concept in which a disulfide bond involved in an active site is specifically reduced and labeled with reactants having no affinity for this site. We constructed a site-directed disulfide reducing agent able in the one hand to specifically recognize the active site of the protein and on the other to reduce the adjacent disulfide bond. A well-documented model for testing this concept is the disulfide bond located between Cys-192 and Cys-193 of the α-subunit of the nicotinic acetylcholine receptor (AChoR) [1,2]. These residues are involved in the binding site of acetylcholine and have already been labeled, after reduction with DTT, with an agonist (BAC) [3–7] or an antagonist (MBTA) [8–10].

This paper describes the use of an analogue of carbamylcholine (ARA) harbouring a readily oxidizable dithiol, with an affinity constant of 20 μM (in its oxidized state) for the agonist binding site of the AChoR, able to specifically reduce the Cys-192–Cys-193 bond on the α-subunits. The kinetic study of this reduction showed a much faster initial rate for ARA than for DTT at the same concentration. The affinity contribution in this difference was confirmed by studying the reduction ability of an ARA analogue where the ethyl-trimethylammonium moiety was replaced by a methyl group.

This site-directed reduction offers the possibility of labeling the nicotinic receptor with a maleimido undecagold cluster and opens up new vistas as to the localization of the acetylcholine binding site in the AChoR, by electron microscopy.

2. Materials and methods

The synthesis of the site-directed disulfide reducing agent (ARA) was described elsewhere [11].

2.1. AChoR preparation

AChoR membrane fragments were prepared from *Torpedo marmorata* electric organs as previously described [12]. Receptor membranes were purified by alkali treatment according to Neubig et al. [13] for their use in labeling experiments with [^{3}H]NEM. In this case, the membrane suspension consisted of 1–3 nmol of toxin binding sites per mg of proteins. Free sulfhydryls were alkylated by excess NEM (5 mM) for 45 min at room temperature followed by dialysis against 50 mM phosphate buffer, pH 7.4, 50 mM NaCl (P buffer). After alkylation, the receptor was desensitized by incubation with Proadifen (15 mM, 1 h).

2.2. Apparent affinity constants determination of oxydized ARA for AChoR

The association kinetics of [^{3}H]α-toxin to AChoR with increasing concentrations of oxydized affinity reagent were determined by following the method previously described [14,15], in presence or not of a non-competitive blocker, and allowed us to determine the apparent inhibition constants ($K_{i\,\alpha\text{-tox}}$) of the reducing agent on the acetylcholine binding site for the receptor in the resting and desensitized states.

The affinity constant ($K_{i\,PCP}$) for the non-competitive blocker site was determined by competition between [^{3}H]phencyclidine (Dositek, France; 50 Ci/mmol, 1 nM) and the oxidized form of the reducing agent (concentration range between 1 μM and 5 mM) after 30 min incubation with carbamylcholine (0.1 mM).

2.3. Labeling of AChoR with [^{3}H]NEM after reduction with the affinity reducing agent or with DTT

Receptor preparations were diluted in degassed P buffer to about

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Dedicated to the memory of Prof. Christian Hirth.

Abbreviations: AChoR, nicotinic acetylcholine receptor; ARA, affinity reducing agent (site-directed disulfide reducing agent); MeRA, methyl analogue of the affinity reducing agent; DTT, dithiothreitiol; NEM, *N*-ethylmaleimide; BAC, bromoacetylcholine; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium; PCP, phencyclidine; Carb, carbamylcholine.

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0.4 mg/ml (determined by the method of Lowry [16]). The reducing agent was added at different concentrations and was allowed to react for 20 min with ARA and for 30 min with DTT. Membranes were pelleted in Eppendorf tubes for 15 min at 17,600 × g and resuspended in the same buffer. [^{3}H]NEM (1 Ci/mmol, 1 mM) was then added to a final concentration of 20 μM for 1 h at room temperature. Following another centrifugation, the pellets were resuspended in P buffer in order to obtain protein concentrations of about 1.4 mg/ml and incubated for 3 h at room temperature in denaturating buffer. 38 μg of proteins were subjected to SDS-PAGE which was performed according to Laemmli [17] on a 10% polyacrylamide gel. After staining (Coomassie brillant R-250) and destaining, the gel was cut into 1 mm slices which were digested with Lipoluma-Lumasolve solution (Luma-LSC, The Netherlands) and counted for tritium radioactivity.

2.4. Protection experiments

Assays were made by incubating carbamylcholine (200 μM) prior to the reduction step. Other protection experiments were carried out with NEM (20 μM) or maleimido undecagold cluster (20 μM) incubated after the reduction step, prior to the alkylation with [^{3}H]NEM.

2.5. Quantification of toxin binding sites in native, reduced, and reduced-alkylated receptor

The concentration of toxin binding sites was measured after incubation of 15 pmol of [^{3}H]α-toxin (tritiated in our laboratory at a specific radioactivity of 27 Ci/mmol) with AChoR (0–10 pmol) in 500 μl of Ringer buffer. After 1 h, 2 × 200 μl was filtered through HAWP filters (Millipore), and after drying under light and addition of scintillating solution (Lipoluma-LSC, The Netherlands) the radioactivity was counted.

Acetylcholinesterase activity, measured by the method of Ellman [18], was inhibited by the addition of Tacrine (5 μM).

For protection experiments, NEM (20 μM) or gold cluster (20 μM) were added to the affinity-reduced membranes prior to the determination of toxin sites.

2.6. Kinetic experiment

Reduction with ARA, DTT or MeRA was carried out as described above; aliquots of the reduced receptor were removed at different times and blocked with an excess of BAC (10 mM) prior to the quantification of α-toxin binding sites. The kinetics of loss of toxin binding sites was inversely proportional to the disulfide bond reduction.

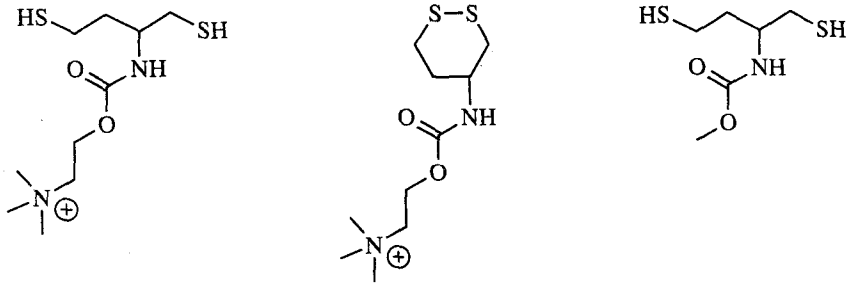
3. Results

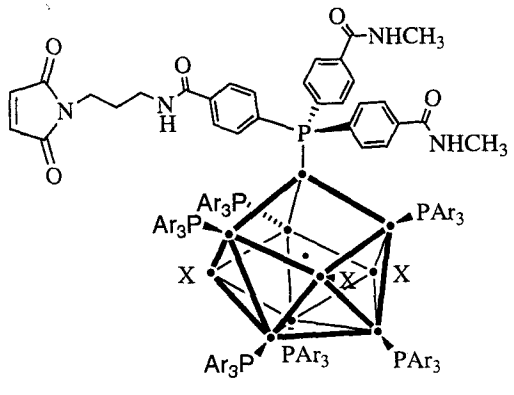
3.1. Synthesis of ARA and MeRA

ARA (Scheme 1) was synthesized as described earlier [11]. MeRA (Scheme 1) was obtained in 62% yield by coupling 4-amino-1,2-dithiane [11] with methylchloroformate in dry tetrahydrofurane.

3.2. Apparent affinity constant determination of the oxidized form of ARA and MeRA for AChoR

The affinity for the acetylcholine binding site was determined from measurements of the inhibition of the initial rate of toxin binding on the nicotinic receptor, using increasing concentrations of ligand. In the receptor desensitized state (in the presence of the non-competitive blocker proadifen) a constant $K_{i\,\alpha\text{-tox}}$ of 20 μM was measured for the oxidized form of ARA (Scheme 1). This was 10 times higher than the constant measured with the receptor in its resting state (data not shown). For the methyl analogue (MeRA), the affinity constant for the





MALEIMIDO-UNDECAGOLD CLUSTER

Scheme 1. Chemical structures of ARA, oxidized ARA, MeRA and maleimido undecagold cluster.

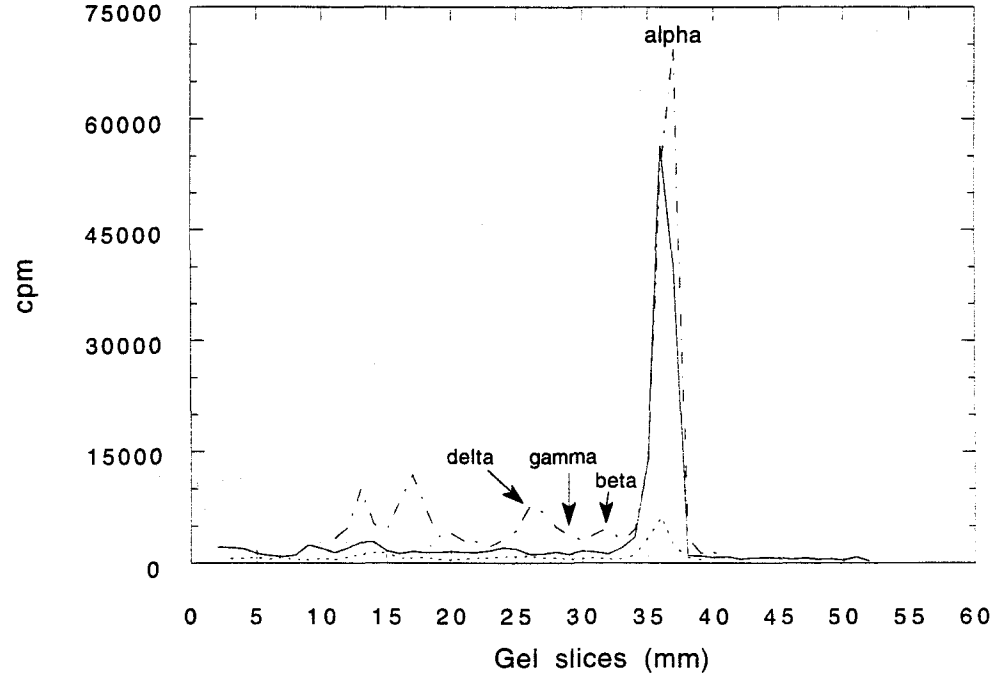


Fig. 1. Alkylation of AChoR membranes by [^{3}H]NEM after reduction with the affinity reducing agent or DTT. Alkali-treated membranes were reduced by ARA (—, 20 μM) or DTT (---, 20 μM: - · -, 200 μM) for 30 min at room temperature. After centrifugation and resuspension in phosphate buffer the membranes were incubated with [^{3}H]NEM (1 Ci/mmol) at 20 μM. Electrophoresis was run on 10% polyacrylamide gels and the gel cut into 1 mm slices, digested and the radioactivity counted.

desensitized receptor was greater than 1 mM. The affinity for the non-competitive blockers site was determined by competition with [^{3}H]phencyclidine in the presence of the agonist carbamylcholine, which induced the desensitized state (highest affinity for the ligands). An affinity constant $K_{i\,PCP}$ of about 1 mM was obtained for ARA and showed its specificity for the cholinergic site.

3.3. Labeling of the reduced AChoR by [^{3}H]NEM

When alkaline-treated membranes were reacted with [^{3}H]NEM (1 mM) after disulfide reduction with the affinity reducing agent at a concentration of 20 μM, the electrophoresis profile of labeled proteins showed an exclusive incorporation of radioactivity on the α-subunit (Fig. 1). No significant labeling of the β-, γ- or δ-subunits occurred.

When the receptor was incubated with an excess of ARA (up to 0.2 mM), the labeling on the α-subunit did not increase in comparison with the assay performed with 20 μM reagent, however, non-specific reduction on other proteins appeared. Furthermore, concentrations lower than 10 μM caused a decrease in [^{3}H]NEM alkylation on the α-subunit, explained by a lower occupancy of the agonist binding site (data not shown).

If the reduction was made in the presence of 20 μM DTT instead of ARA, the incorporation of radioactivity on the α-subunit represented only 15% of the labeling after reduction with the affinity reagent. Furthermore, a concentration of DTT 10 times higher (0.2 mM) was necessary to observe a comparable labeling on the α-subunit by [^{3}H]NEM, but in this case the radioactive background was substantially more important and non-α-subunits and non-receptor proteins were labeled (Fig. 1). The relatively important labeling on the δ-subunit could be explained by the partial reduction of the dimeric form of the AChoR which is present in the membrane preparation and which involves a disulfide bond between two δ-subunits.

Incubation of the reduced receptor with thiol reagents, i.e non-radioactive NEM or maleimido undecagold cluster (Scheme 1) prior to [^{3}H]NEM labeling totally abolished the incorporation of radioactivity (Fig. 2). A protection experiment was carried out with carbamylcholine (preincubated at 200 μM) in which an 80% decrease in the α-subunit labeling was observed (Fig. 2), demonstrating the specificity of the reduction step.

3.4. Kinetic of the disulfide bond reduction by ARA, DTT or MeRA

In order to confirm the affinity step for ARA reduction, a kinetic study of the disulfide bond reduction by the different reducing agents was carried out. At different times, the free sulfhydryls were reacted with an excess of bromoacetylcholine (BAC, 10 mM) and the residual α-toxin binding sites were quantified. These experiments showed that the time–course that is required to completely reduce the disulfide bond adjacent to the ligand binding site was about 5 min in the case of ARA (20 μM), 20 min for DTT (200 μM), in agreement with previous reports [4], and up to 2 h for MeRA or DTT at 20 μM (Fig. 3). The comparison of the initial rate of reduction between ARA, MeRA and DTT (Fig. 3) showed clearly the involvement of an affinity step of ARA which reacted about 25 times faster on the disulfide bond than DTT or MeRA at the same concentration (20 μM). At 200 μM, the initial rate of reduction with DTT was still nearly 4 times lower than with ARA at 20 μM.

No loss of toxin binding sites was observed after DTT reduction or alkylation with NEM or maleimido undecagold cluster. However, a loss of about 35% of the binding sites was observed

in the case of ARA-reduced receptor in comparison with non-reduced or DTT-reduced ones. This loss could be explained by the formation of mixed disulfides between the cysteines from the receptor and the reducing agent. When ARA reduction was followed by DTT reduction the toxin binding sites were recovered (data not shown).

4. Discussion

In this paper, we describe a new approach for the site-directed reduction of disulfide bonds of the AChoR using a site-directed disulfide reducing agent. We built a carbamylcholine analogue (ARA) harbouring a dithiol which forms, like DTT, a favorable 1,2-dithiane upon oxydation. The lower affinity for the AChoR of the reducing agent (20 μM, in its oxidized form) as compared to carbamylcholine (K_i = 0.5 μM [15]) could be due to the addition on the polar carbamide end of carbamylcholine of a hydrophobic non-polar reducing moiety.

The exclusive labeling of the α-subunit by [^{3}H]NEM following the site-directed reduction with ARA (Fig. 1) can be compared with previous reports in which a non-specific reducing agent (DTT) was used and the cysteinyl residues in the active site were labeled with an affinity probe (MBTA or BAC [1–10]). However, the site-directed reduction with ARA required both a reduced time and a lower concentration of reducing agent (Figs. 1 and 3). It allowed the selective labeling of cystinyl residues of the functional sites by thiol reagents having no affinity for that site. Furthermore, considering the size of our reagent, our data confirmed the previous proposals that the disulfide Cys-192–Cys-193 bond is located at about 1 nm from the quaternary ammonium binding site [1,2,10]. Indeed, reduction experiments carried out with an ARA analogue possessing a methylene group between the carbonyl and the nitrogen of the carbamylcholine moiety showed no affinity process for this reducing agent, and the reduction proceeded in the same way as with MeRA or DTT (20 μM) (data not shown).

The involvement of a specific recognition step in the reduction process with ARA was shown by the protective effect of carbamylcholine on this reduction (Fig. 2) and by the characteristic kinetic properties of ARA in comparison with DTT or MeRA (Fig. 3). The fast initial rate of reduction with ARA could be explained by the high local concentration of reducing agent in proximity to the disulfide bond of the receptor, due to the affinity property of ARA, which is not the case for its methyl analogue (MeRA) or for DTT. Hence, the site-directed reduction leads to a more specific labeling of the α-subunit (no radioactivity incorporated in non-α-subunits) using a 10 times lower concentration of reducing agent.

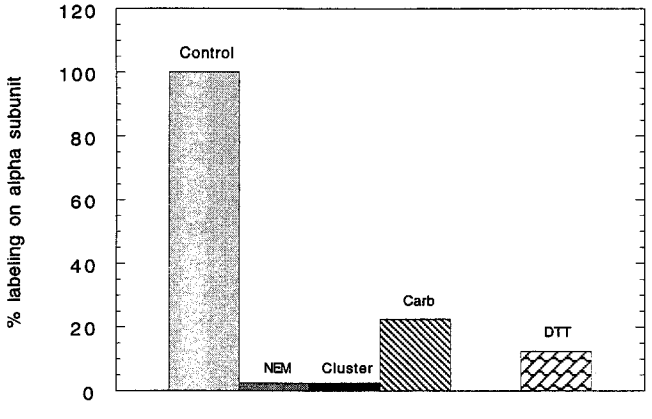


Fig. 2. Specificity of the site-directed reduction. Alkali-treated membranes were treated as in Fig. 1. The membranes were incubated for 30 min either with carbamylcholine (200 μM) prior to the reduction with ARA or with NEM (20 μM) or maleimido gold cluster (20 μM) prior to the alkylation with [^{3}H]NEM. The amount of radioactivity incorporated into the α-subunits was compared to the control experiment (as in Fig. 1). The labeling after reduction with DTT (20 μM) was obtained with the same protocol.

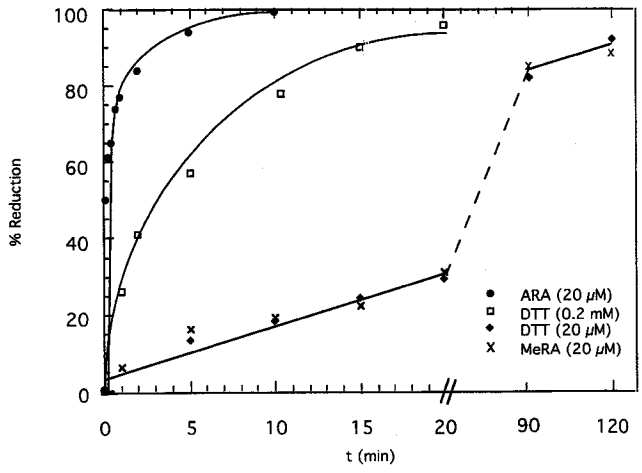


Fig. 3. Time-course reduction of the disulfide bond by ARA, MeRA or DTT. Aliquots of the receptor incubated with the different reducing agents were removed at different times, blocked with excess BAC (10 mM) and used to determine the residual toxin binding sites as described in section 2.

It should be noted that preliminary experiments made with higher DTT concentrations (2 or 20 mM) induced a significant increase in radioactivity incorporation in the α-subunit compared with DTT (0.2 mM) or with the affinity reducing agent (20 μM) (data not shown). Possibly, the other extracellular disulfide bond between Cys-128 and Cys-142 on the α-subunit was thus reduced only at high concentrations of DTT. No such reduction occurs in the presence of ARA. At this high DTT concentrations, labeling appeared on non-α-subunits and in particular on the δ-subunit which has an additional extracellular disulfide bond with a neighbouring receptor. The reduction of disulfide bonds depends on the concentration of reducing agent. In the case of the AChoR, the vicinal cysteine–cysteine bond was shown to be much more fragile than other cysteines, and hence it is possible to reduce this bond almost exclusively using appropriately low concentrations of DTT (Fig. 1). Such selectivity may not always be observed in the case of other receptors or enzymes possessing several accessible disulfide bonds.

However, after the ARA reduction, about 35% of the toxin binding sites were lost compared to the non-reduced or DTT-reduced receptor. This loss was probably due to mixed disulfides between the receptor and the reactant, confirmed by the recovery of all these sites by an additional DTT reduction. The synthesis of new carbamylcholine analogues is now in progress to avoid the formation of disulfide bridges between the receptor and the reducing ligand.

In a previous report [19], we described the stereospecific

monomodification of a snake toxin with maleimido undecagold cluster for the determination of the three-dimensional localization of its binding site on the AChoR. We have now specifically reduced a disulfide bond in the vicinity of the agonist binding site and labeled it with the same cluster. This labeling inhibited the incorporation of [^{3}H]NEM in the cysteinyl residues (Fig. 2). These preliminary results open the way for the labeling of the acetylcholine binding site by an electron-dense cluster, making it available for study by electron microscopy. This could be particularly helpful to confirm previous reports [20] that postulated that the acetylcholine binding site was about 30 Å from the bilayer surface on the α-subunit, and it should help to localize the site in the central pit or on the external surface of the AChoR.

The AChoR reduction by the site-directed reducing agent will also be an interesting tool for the use of aromatic arsenoxides as selective labeling reagents for the nicotinic receptor. In fact, the biological effect of arsenicals was previously investigated [21] and particularly for labeling the AChoR by forming covalent yet reversible complexes with pairs of thiols [22,23]. The use of an affinity reducing agent which specifically reduces the disulfide bond at the vicinity of the agonist binding site could greatly increase the efficacy of the arsenical labeling and so the detection of many subtypes of neuronal and muscular AChoR.

This new concept of selective disulfide bond reduction could probably be extended to other receptors or enzymes where a disulfide bond is suspected to be involved in or near the ligand binding site or catalytic site. For instance, the glycine receptor is, along with the nicotinic receptor, a member of the ligand-gated ion channel group which possesses two extracellular disulfide bonds on the α-subunit. It could be possible to investigate with appropriately derivatized strychnine the proximity of a disulfide bridge to the antagonist binding site.

Furthermore, for biochemical studies on AChoR using cell lines, site-directed disulfide reducing agents could help to avoid the cytotoxic effects of the more usual reductants such as DTT.

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