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# Reaction of Both Active Site Thiols of Reduced Thioredoxin Reductase with *N*-Ethylmaleimide†

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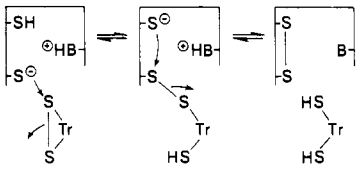
ABSTRACT: Thioredoxin reductase from *Escherichia coli*, only in its reduced state, reacts rapidly with 2 mol of *N*-ethylmaleimide, which specifically alkylates both active site cysteine residues. This dual modification supports previous studies indicating that a base lowers the p*K* of both active site cysteine residues. The dual modification also indicates that the region around the active site dithiol is more open than is the case with the related enzymes lipoamide dehydrogenase and glutathione reductase, both of which can be alkylated only on one nascent thiol. Enhanced nucleophilicity of the active site thiols is consistent with the proposed chemical mechanism of thioredoxin reductase. The sequence of the amino-terminal 16 residues is presented.

Thioredoxin reductase from *Escherichia coli* catalyzes the reversible transfer of electrons between NADPH[1] and the disulfide of thioredoxin, a small protein (Moore et al., 1964). The active center of thioredoxin reductase contains a FAD and an oxidation–reduction active disulfide (Zanetti & Williams, 1967; Thelander, 1968). It is thought that the electrons flow from NADPH to the FAD, from the FAD to the disulfide, and from the dithiol to the disulfide of thioredoxin. On the basis of model studies, electrons would be transferred from the active site dithiol moiety on thioredoxin reductase to the disulfide of thioredoxin in two steps (Scheme I): (1) nucleophilic attack by one enzyme thiol on the disulfide of thioredoxin to form a mixed disulfide bridge between enzyme and substrate and (2) nucleophilic attack by the other enzyme

Scheme I: Roles of the Active Site Thiols of Thioredoxin Reductase in the Reduction of Thioredoxin

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[1] Abbreviations: NEM, *N*-ethylmaleimide; APADP$^+$, oxidized 3-acetylpyridine adenine dinucleotide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; DTE, 2,3-dihydroxy-1,4-dimercaptobutane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; TNB, 5-thio-2-nitrobenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; dpm, disintegrations per minute.

active site thiol on the mixed disulfide to generate a disulfide on thioredoxin reductase and release reduced thioredoxin. Model studies show nucleophilic attack of a thiol on a disulfide is via the thiol anion species (Foss, 1961). The thiol of an isolated cysteine residue exposed to solvent has a p$K$ value above 9.0 (Jocelyn, 1972), yet thioredoxin reductase is highly active at pH 7 (Moore et al., 1964). Two independent studies indicate a base at the active site that interacts with either thiol lowering their effective p$K$ values (O'Donnell & Williams, 1983, 1984).

The activated double bond of NEM[1] alkylates protein sulfhydryl groups, and at pH values less than 7.0 it is quite specific for thiols (Smyth et al., 1964). Nucleophilic attack on NEM is via the thiol anion species (Semenow-Garwood, 1972). Thus, only the most reactive protein thiols are modified by NEM. In this report activation of the active site thiols in thioredoxin reductase is tested directly by reaction with NEM.

## Experimental Procedures

*Materials.* Thioredoxin reductase was purified by a modification (O'Donnell & Williams, 1983) of the method of Pigiet & Conley (1977). NEM from Sigma was further purified by sublimation. *N*-Ethyl[2,3-$^{14}$C]maleimide from Amersham was diluted with unlabeled NEM to a specific activity of 39 000 dpm/nmol. NADPH and APADP$^+$ were from Sigma. TPCK–trypsin was from Worthington. Sequenator chemicals were from Beckman. Acetonitrile (UV spectral grade) was from Burdick and Jackson. All other chemicals were analytical reagent grade. The buffers used in these studies were appropriate mixtures of sodium dihydrogen phosphate and potassium hydrogen phosphate (buffer).

*NEM Concentration.* The concentration of [$^{14}$C]NEM (before dilution with unlabeled NEM) and unlabeled NEM solutions were determined by absorbance ($E_{305}$ = 620 M$^{-1}$ cm$^{-1}$) and by thiol reactivity; the two methods agreed with 5%. Concentration measurement by thiol reactivity was calculated from the loss of absorbance at 412 nm upon adding approximately 25 nmol of NEM to a solution containing the TNB anion ($E_{412}$ = 14 150 M$^{-1}$ cm$^{-1}$); the absorbance change occurred with a half-time of approximately 1 min. The TNB anion was formed in a quartz cuvette (0.2-cm path length) by addition of 0.16 μmol of DTE to 1.8 μmol of DTNB in 0.61 mL of 0.1 M buffer and 0.3 mM EDTA, pH 7.0.

*Spectral Characterization of NEM-Modified Thioredoxin Reductase.* Anaerobic titrations with dithionite and NADPH were performed as described by O'Donnell & Williams (1983), and spectra were recorded on a Cary 118C spectrophotometer (Williams et al., 1979).

*Assays.* The native and NEM-modified enzymes were assayed at 25 °C by the DTNB-coupled assay, which measures FAD-mediated electron transfer between NADPH and thioredoxin (Moore et al., 1964), and the transhydrogenase NADPH/APADP$^+$ assay, which measures FAD-mediated electron transfer between pyridine nucleotides (Thorpe & Williams, 1976).

*Trypsin Digest.* The dialyzed protein was denatured in 6 M guanidine hydrochloride containing a 10-fold molar excess of DTE over total protein thiols and 10 mM EDTA in a 5-h incubation at pH 7.8 (40 °C) under a $N_2$ atmosphere prior to the addition of recrystallized iodoacetate (2-fold molar excess over total thiols in the reaction mixture). After a 30-min incubation in the dark, the solution was acidified and dialyzed against 1% ammonium bicarbonate. The dialyzed protein solution was digested with 2% (w/w) TPCK–trypsin for 4 h at room temperature. Comparison of HPLC peptide maps of samples taken at various times of digestion showed the digestion was complete after 2 h.

*Peptide Isolation and Characterization.* Analytical peptide mapping was performed by reverse-phase HPLC as described by Fullmer & Wasserman (1979). A total of 1–2 nmol of protein digest was analyzed on a Waters Associates μBondapak $C_{18}$ column (0.4 i.d. × 30 cm) at room temperature using a linear gradient from 0.1% phosphoric acid to 50% acetonitrile in 0.1% phosphoric acid over 70 min at a flow rate of 1.9 mL/min. Peptides were detected by their absorbance at 220 nm with the Waters Associates Model 450 detector flow cell. Preparative levels (ca. 30 nmol) of peptides could be purified in this same manner. The radioactive fraction (retention time = 57 min) was further purified on the same reverse-phase HPLC column using a linear gradient from 1 mM buffer, pH 7.4, to 50% $CH_3CN$ in 1 mM buffer, pH 7.4, over 70 min at a flow rate of 1.9 mL/min.

*Sequencing Methodology.* Amino acid sequences were determined by the automated Edman degradation procedure with a Beckman Model 890C sequenator (Tarr, 1985). The repetitive yield for each sequence analysis was determined by linear regression analysis of the logarithms of the yields of all the PTH derivatives except PTH-serine and PTH-threonine vs. cycle number. The initial coupling was determined by extrapolation of these data to the first coupling event.

Amino acid analysis was performed as described previously (Swenson et al., 1982). The color constant used for *S*-succinylcysteine (emerging just ahead of aspartic acid) was 1.08 times that for aspartic acid (Fonda & Anderson, 1969). Three microliters of ethanethiol was contained in the hydrolysis of NEM-modified protein. Phenol was not added to protect against tyrosine destruction during hydrolysis.

## Results

*Preparation and Characterization of NEM-Modified Thioredoxin Reductase.* Oxidized thioredoxin reductase (88 μM) in aerobic buffer did not react significantly with NEM (120 μM) under the conditions used. The enzyme spectrum and activity were not altered over the 40-min reaction time, and the slight change in NEM absorbance at 305 nm indicated only 0.1 mol of NEM had reacted per mole of enzyme–FAD (data not shown). This supports a previous observation that the other four thiols per subunit (in addition to the redox-active thiols) are unreactive with iodoacetate (Thelander, 1968).

A solution of thioredoxin reductase that is half-reduced (two electrons) is an equilibrium mixture of four species: the oxidized form, two-electron reduced enzyme (disulfide, $FADH_2$), two-electron reduced enzyme (dithiol, FAD) and four-electron reduced enzyme (O'Donnell & Williams, 1983). Addition of NEM to an anaerobic, partially reduced solution of thioredoxin reductase causes the reappearance of FAD absorbance with a half-time of approximately 6 min (Figure 1). Upon aeration, the enzyme regained full absorbance and showed a 2-nm blue shift of both visible peaks (Figure 1). The dialyzed enzyme had 15% of the activity of native enzyme in the DTNB-coupled assay. The fact that oxidized enzyme is unaffected by NEM while partially reduced enzyme is inactivated by NEM and regains FAD absorbance during reaction indicates alkylation of an active site thiol (or thiols). Residual activity can be accounted for by unmodified fully oxidized enzyme present in the partially reduced enzyme during reaction with NEM.

To achieve more complete reaction with NEM, thioredoxin reductase (300 nmol) was fully reduced with a 10-fold excess of NADPH (enzyme–FAD) under anaerobic conditions in 2.4 mL of 0.1 M buffer, pH 7.0, and 0.3 mM EDTA, prior to NEM addition (5-fold excess per enzyme–FAD) at 12 °C. The enzyme was inactivated over 99% within 15 min, the

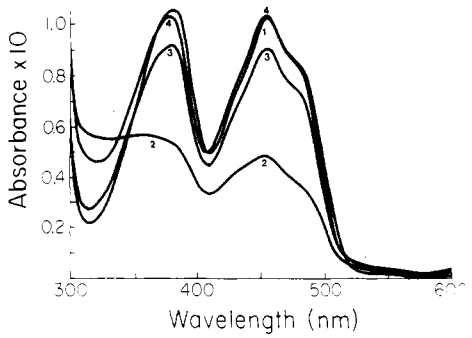


FIGURE 1: Spectra observed upon reaction of NEM with thioredoxin reductase. An anaerobic solution of thioredoxin reductase (10.6 nmol of enzyme–FAD in 1.2 mL of 0.1 M buffer, pH 7.0, and 0.3 mM EDTA at 12 °C (spectrum 1), was reduced by 1.2 equiv of dithionite (spectrum 2). Spectrum 3 was obtained upon reaction with 16 nmol of NEM. Spectrum 4 was produced upon aeration.

minimum time required for the anaerobic manipulations and the assay. After dialysis the NEM-modified enzyme remained inactive in the DTNB-coupled assay (which requires participation of the active site thiols) but retained 95% activity in transhydrogenation from NADPH to APADP$^+$.

Native enzyme requires 2 equiv of sodium dithionite for reduction of the enzyme–FAD and disulfide (O'Donnell & Williams, 1983). The FAD in NEM-modified thioredoxin reductase was completely reduced upon reaction with 1.2 equiv of sodium dithionite in an anaerobic titration (Figure 2A), consistent with prior modification of the redox-active thiol(s). Likewise, the equilibrium between NEM-modified enzyme and NADPH extrapolates to full reduction by 1.24 equiv (Figure 2B). Reaction of the modified enzyme with NADPH was rapid as expected from the transhydrogenase activity.

*Stoichiometry.* Titration of NEM into an anaerobic solution of thioredoxin reductase reduced with a 10-fold excess (enzyme–FAD) of NADPH showed 98% loss of the DTNB-coupled activity with 1.5 mol of NEM per mole of enzyme–FAD (Figure 3). Reaction of one thiol per enzyme molecule should be sufficient to eliminate the activity (Scheme I). The stoichiometry of inactivation may be explained by the reaction of either active site thiol with NEM.

Reaction of a 2.2-fold excess of [$^{14}$C]NEM with reduced thioredoxin reductase completely inactivated the enzyme within 40 min. Upon extensive dialysis the NEM-modified enzyme retained 1.84 mol of [$^{14}$C]NEM per mole of enzyme–FAD. Since oxidized enzyme reacts with less than 0.1 mol of NEM per mole of enzyme–FAD, the amount of radiolabel indicates reaction of NEM with both active site thiols. The possibility that a thiol outside the active site becomes activated upon reduction of the redox-active disulfide cannot be ruled out.

*Isolation and Characterization of the NEM-Modified Tryptic Peptide.* Complete digestion of denatured and carboxymethylated NEM-modified thioredoxin reductase with TPCK–trypsin produced at least 24 peptide peaks (39 Lys and 16 Arg per enzyme–FAD) upon HPLC chromatography (Figure 4A). The HPLC profile was unchanged by further digestion or addition of more trypsin. The pH 2.0 HPLC peptide map was reproducible with over 90% recovery of the injected radioactivity. Approximately 80% of the eluted radioactivity is in one peak (retention time = 57 min), indicating a highly specific reaction with NEM. Further purification of the 57-min peak was achieved upon reinjection on the same reverse-phase column at pH 7.4 (Figure 4B), and over 80% of the injected radioactivity was in a 53-min peak; material in this peak will be referred to as the NEM-peptide. Its amino acid analysis (in nmol) was as follows: Asp, 8.1; Thr, 7.3; Ser, 8.2; Gly, 20.1; Ala, 19.8; Val, 10.7; Tyr, trace; Phe, 17.7; Arg, 6.7; *S*-succinylcysteine, 6.5.

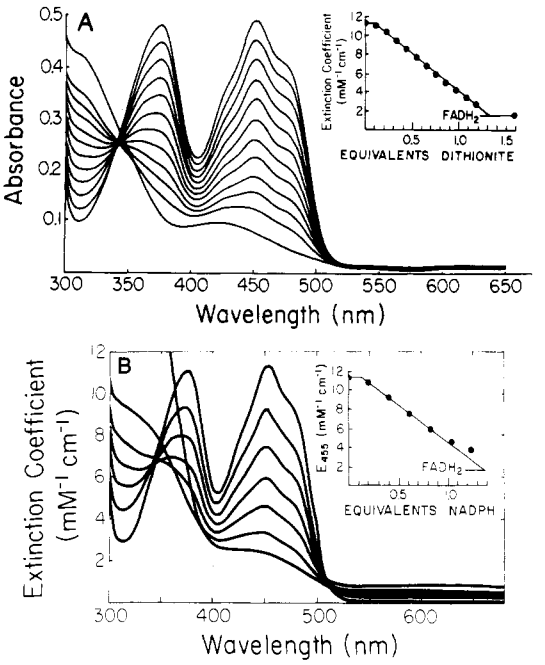


FIGURE 2: Spectra observed upon anaerobic reduction of NEM-modified thioredoxin reductase titrated with (A) dithionite and (B) NADPH. Panel A is an anaerobic dithionite titration of NEM-modified thioredoxin reductase (53 nmol) in 1.2 mL of 0.1 M buffer, pH 7.6, and 0.3 mM EDTA at 12 °C. The solid curves from top to bottom represent oxidized enzyme and 0.22, 0.32, 0.43, 0.54, 0.65, 0.75, 0.86, 0.97, 1.08, 1.18, and 1.61 equiv of dithionite/mol of FAD. Inset: Relationship of the extinction coefficient at 456 nm to equivalents of dithionite added. Residual oxygen accounts for 0.1 equiv of dithionite/mol of FAD. The curve corresponding to the point at 0.11 equiv has been omitted for clarity. Panel B is a NADPH titration of 21.2 nmol of NEM-modified thioredoxin reductase in 1.2 mL of 0.1 M buffer, pH 7.6, and 0.3 mM EDTA at 12 °C. The solid curves from top to bottom represent oxidized enzyme and 0.42, 0.63, 0.84, 1.05, 1.47, and 1.78 equiv of NADPH/mol of FAD. Inset: Relationship of the extinction coefficient at 455 nm to equivalents of NADPH added. Residual oxygen accounts for 0.14 mol of NADPH/mol of FAD.

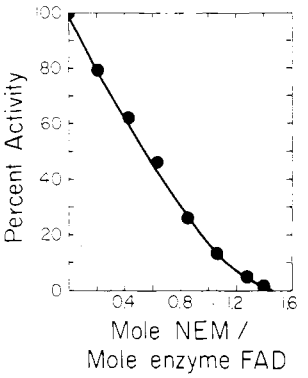


FIGURE 3: Relationship of NEM concentration to thioredoxin reductase activity. Thioredoxin reductase (45 nmol) in 0.5 mL of 0.1 M buffer, pH 7.0, and 0.3 mM EDTA was made anaerobic, reduced by addition of 450 nmol of NADPH, and then reacted with the indicated amount of NEM for 40 min at 12 °C. Data points are separate experiments. The reaction mixture was diluted 10 000-fold into the DTNB-coupled assay.

The yield of *S*-succinylcysteine in this analysis indicates that degradation took place in the hydrolysis, assuming reaction of both active site thiols with NEM. This is based on the following reasoning: 1.84 equiv of NEM were bound per mole of enzyme–FAD, and Figure 4 showed that virtually all of the

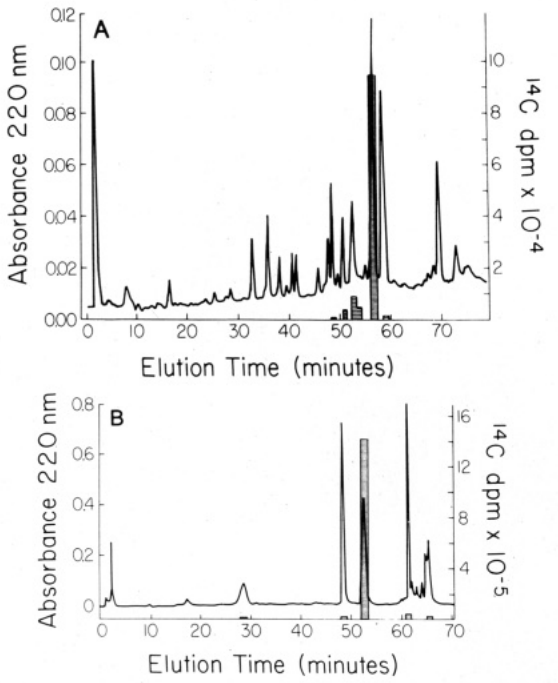


FIGURE 4: Purification of the NEM-modified tryptic peptide by HPLC chromatography. Panel A is a low-pH HPLC chromatogram of NEM-modified thioredoxin reductase digested with TPCK–trypsin. Approximately 2 nmol of a tryptic digest of [$^{14}$C]NEM-modified thioredoxin reductase (see text) was chromatographed on a Waters Associates $\mu$Bondapak $C_{18}$ column using a linear gradient from 0.1% phosphoric acid to 50% acetonitrile in 0.1% phosphoric acid over 70 min (flow rate, 1.9 mL/min). Peptide elution times monitored by absorbance at 220 nm and a histogram of the radioactivity determined for each peak are shown. Panel B is a high-pH HPLC chromatogram of the radioactive 57-min peak. Approximately 15 nmol of the 57-min peak from a preparative-scale low-pH column (acetonitrile removed by evaporation) in 1.0 mL of 1.0 mM buffer, pH 7.4, was applied to a Waters Associates $\mu$Bondapak $C_{18}$ column and eluted with a linear gradient from 1 mM buffer, pH 7.4, to 50% acetonitrile in 1 mM buffer, pH 7.4, over 70 min (flow rate, 1.9 mL/min).

bound radioactivity was associated with the peptide. Furthermore, of the 742 000 dpm applied to the amino acid analyzer only 290 000 dpm were associated with the *S*-succinylcysteine peak. Assuming that there was degradation during the hydrolysis, the counts would indicate that there was 16.7 nmol of *S*-succinylcysteine relative to the other amino acids. That both cysteines in this peptide were partially labeled was borne out by the sequencing data shown below.

Sequence analysis of 14 nmol of NEM-peptide by automated cycles of Edman degradation showed a 14 amino acid peptide. Radioactivity in each Edman degradation cycle was measured to determine the site(s) of modification by [$^{14}$C]-NEM (total radioactivity placed in the cup was 16 700 dpm). Two peaks of radioactivity were observed, cycles 5 and 8, which contained equal amounts of radioactivity after correcting for the repetitive yield. Residues 5 and 8 appeared fully modified, as HPLC chromatography of these cycles showed only one major peak (PTH derivative) having a retention time different from the PTH derivatives of the common amino acids; no other peak was apparent over background in these cycles. The sequence of this peptide is

-Gly-Val-Ser-Ala-Cys-Ala-Thr-
Cys-Asp-Gly-Phe-Phe-Tyr-Arg-

and extends the previously published active site sequence by three residues in both directions (Thelander, 1970; Ronchi & Williams, 1972).

*Amino-Terminal Analysis of the Apoprotein.* It has been found for many flavoproteins that the binding site for FAD is formed from the amino-terminal portion of the polypeptide chain. There is a sequence of apolar residues and glycine residues that is characteristic of the $\beta$–$\alpha$–$\beta$ structure of the pyridine nucleotide binding site (Rossmann et al., 1975). Automated Edman degradation of 27 nmol of the apoprotein indicated the sequence

Gly-Thr-Thr-Lys-His-Ser-Lys-
Leu-Leu-*Ile-Leu-Gly*-Ser-*Gly*-Pro-Ala-*Gly*-

The trxb gene encoding thioredoxin reductase has been cloned (Russel & Model, 1985), and the nucleotide sequence is being determined. The amino-terminal amino acid sequence will confirm the reading frame.

## Discussion

Electron transfer from the dithiol of thioredoxin reductase to the disulfide of thioredoxin requires both enzyme active site thiols to be nucleophilic in turn (see Scheme I). Inasmuch as thiol attack on a disulfide is initiated by the thiol anion species (Foss, 1961) and the enzyme catalysis is efficient at pH 7.0, both thiols are proposed to have lowered p$K$ values relative to cysteine in solution (p$K$ = 9.0). Studies on the pH dependency of the oxidation–reduction couples of thioredoxin reductase (O'Donnell & Williams, 1983) and characterization of the apoenzyme reconstituted with 1-deaza-FAD (O'Donnell & Williams, 1984) produced independent evidence for an enzyme base that interacts with both active site thiols to effect their lowered p$K$ values. Since reaction of thiols with NEM is also initiated by the thiol anion species (Foss, 1961), alkylation of both active site thiols by NEM supports the lowered p$K$ values.

Modification of the active site thiols had little effect on the transhydrogenase (NADPH/APADP$^+$) activity. This is expected given the transfers NADPH to enzyme–FAD and enzyme–$FADH_2$ to APADP$^+$ do not involve the disulfide/dithiol couple.

Only one of the nascent thiols of lipoamide dehydrogenase and glutathione reductase, two closely related disulfide flavoproteins, exhibits reactivity toward iodoacetamide (Thorpe & Williams, 1976; Arscott et al., 1981). In both enzymes it is the thiol nearer the N-terminus that reacts; this is the thiol that interchanges with the substrate rather than the thiol that reacts with the flavin. Hence, it is noteworthy that in thioredoxin reductase, both thiols are available for reaction with NEM, indicating a more open active site. The more open active site is perhaps not surprising given that the substrate is a protein.

## Acknowledgments

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# Interaction of Alamethicin with Lecithin Bilayers: A $^{31}P$ and $^{2}H$ NMR Study†

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ABSTRACT: The interaction of alamethicin with artificial lecithin multilamellar dispersions was investigated by nuclear magnetic resonance (NMR) and Raman spectroscopies. $^{31}P$ NMR studies revealed perturbation of the lipid head groups in the presence of the icosapeptide. Simulation of the $^{31}P$ NMR spectra indicated that the observed spectral changes could be attributed to slight variations in the average tilt angle of the head groups. In contrast, no noticeable effect of the peptide on the segmental order of the hydrophobic acyl chains of the lipid molecules was detected by $^{2}H$ NMR and Raman spectroscopic measurements. Taken together, these results indicated that, in the absence of a transmembrane electric potential, alamethicin interacts primarily at the water–lipid interface without significant insertion or incorporation into the bilayer leaflet.

Alamethicin is an antibiotic icosapeptide extracted from the fungus *Trichoderma viride*, which exhibits voltage-gated ionic conductance in black lipid membranes (Latorre & Alvarez, 1981). On account of its structural simplicity, this molecule can serve as an ideal model system to study the mechanism of gated ionic conductance through lipid bilayers. In a recent paper, we reported the structure of alamethicin (Banerjee et al., 1983) in methanol and aqueous methanol mixtures. On the basis of nuclear magnetic resonance (NMR)[1] measurements at 11.74 T, the peptide was shown to exist as a dimer in methanol, stabilized by intermolecular hydrogen bonds at the C-terminus. Dimerization in methanol was confirmed by proton spin–spin relaxation measurements (Banerjee & Chan, 1983). Proton amide coupling constants ($J_{NH-\alpha CH}$) indicate that the N-terminal end of the molecule is $\alpha$-helical over nine residues, while the structure at the C-terminus corresponds to the $\beta$-pleated sheet. An important feature of this dimer is that one face is completely hydrophobic, while the other is lined with polar groups. Studies in water–methanol mixtures revealed that the secondary structure of the peptide remains unchanged in the more polar solvents. Extensive aggregation of the peptide was noted in water, however, and it was proposed that the dimer associates further to yield highly amphipathic micellar structures possibly via sequestering of the hydrophobic domains of the peptide away from water.

In the present paper, we describe a series of studies designed to elucidate the interaction of alamethicin with phospholipid membranes. There has been much debate over the years about the partitioning of the peptide into the bilayer membrane. Early studies have suggested that alamethicin is primarily a surface-active molecule with minimal partitioning into the hydrophobic core of the membrane in the absence of an electric field (Lau & Chan, 1975, 1976). Single channels can then be formed by the insertion of alamethicin into the bilayer upon the application of an electric potential. Others have proposed complete partitioning of alamethicin into bilayer membranes (Fringeli & Fringeli, 1979; Latorre et al., 1981). In fact, models for alamethicin conductance have been proposed which require preaggregation in the membrane (Boheim et al., 1983).

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[1] Abbreviations: NMR, nuclear magnetic resonance; DML, dimyristoylphosphatidylcholine; DML-$d_{54}$, bis(perdeuteriomyristoyl)phosphatidylcholine; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

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