VOLUME 21 NUMBER 12

ACCOUNTS OF CHEMICAL RESEARCH®

DECEMBER 1988

Membrane-Impermeant Cross-Linking Reagents: Probes of the Structure and Dynamics of Membrane Proteins

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Received June 1, 1988 (Revised Manuscript Received August 3, 1988)

Every living cell is bounded by a plasma membrane composed of lipids and proteins, plus a small amount of carbohydrate.[1,2] The lipids form a bilayer that acts as a barrier to the passage of inorganic ions and hydrophilic molecules into or out of the cell. The carbohydrate, which for the most part occurs as a component of glycoproteins or glycolipids, appears to function in processes such as cell–cell recognition. However, for transmembrane processes, i.e., the selective movement of inorganic ions or of hydrophilic organic molecules into or out of the cell or the transmembrane signaling of the binding of a hormone to a receptor on the cell surface, proteins are the key players.

Proteins that carry out transmembrane functions are generally transmembrane in structure, and every copy of a particular protein has the same orientation in the membrane.[2] Early studies of a number of membrane proteins had suggested that these proteins were oligomeric, either made up of different subunits, as in the case of the Na^+, K^+-ATPase,[3] or made up of two or more copies of a single subunit, as in the case of the anion-exchange channel of the erythrocyte.[4] Other studies had demonstrated that many transmembrane proteins can be dynamically associated with fibrous, polymeric protein structures in the cytoplasm of the cell, collectively known as the cytoskeleton or membrane skeleton.

James V. Staros was born in 1947 and began his scientific career some 20 years later, when, as an undergraduate at Dartmouth College, he joined the research group of David Lemal to work on a problem in physical organic chemistry. After graduating from Dartmouth, he moved to Yale University as an NSF Graduate Fellow. He received the Ph.D. from Yale in 1974 in molecular biophysics and biochemistry, having worked under the tutelage of Frederic Richards. Next he moved to the Department of Chemistry at Harvard University, where he was a Helen Hay Whitney Postdoctoral Fellow with Jeremy Knowles. In 1978, he moved to Vanderbilt University, where he is presently Professor of Biochemistry and thoroughly enjoying life at the interface of chemistry and biology.

Membrane proteins that are deeply embedded in the lipid bilayer cannot, for many purposes, be manipulated in the same manner as soluble proteins. In particular, solubilization requires disruption of the bilayer, usually with detergents. The presence of detergents, in turn, renders many physical methods difficult or impractical. For this reason, chemical cross-linking has been an important approach for studying associations between subunits of oligomeric membrane proteins and between membrane and cytoskeletal proteins.[5,6]

Investigations of quaternary structure of oligomeric membrane proteins differ in another important respect from such studies of soluble oligomeric proteins: interactions between subunits may occur in the cytoplasmic (intracellular) and/or the extracytoplasmic (extracellular) domain of the oligomer. Ideally, one would like to obtain this topological information as well as the proximity information usually associated with cross-linking experiments. These considerations led to the development of a series of membrane-impermeant cross-linking reagents, which, in turn, led to the preparation of reactively bifunctional spectroscopic probes. In this Account, I shall briefly describe the stategies employed in the design of such reagents and some of the information that can be obtained with them.

Strategies

The first membrane-impermeant cross-linking reagent was the bis(alkyl imidate) diisethionyl 3,3′-dithio-

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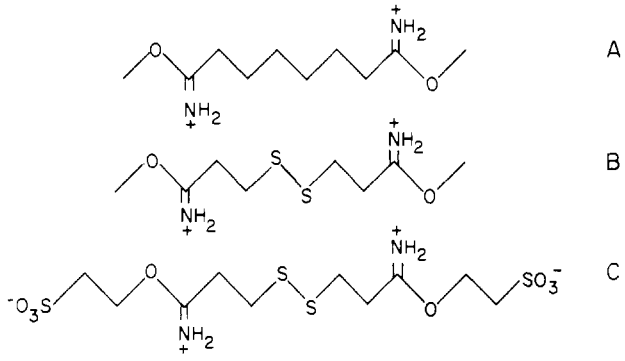

Figure 1. Alkyl imidate cross-linking reagents dimethyl suberimidate (A), dimethyl 3-3′-dithiobis(propionimidate) (B),[5] diisethionyl 3,3′-dithiobis(propionimidate) (C).[7]

bis(propionimidate),[7] which was designed as a membrane-impermeant analogue of dimethyl 3,3′-dithiobis(propionimidate),[5] which itself is an analogue of dimethyl suberimidate (Figure 1). Alkyl imidates have been widely employed in cross-linkers and other protein modification reagents because of their specificity for amino groups and because of the ionizable character of the product amidine:

$$R^1\text{-}NH_2 + R^2\text{-}C(=NH_2^+)\text{-}OR^3 \longrightarrow R^1\text{-}NH\text{-}C(=NH_2^+)\text{-}R^2 + R^3OH$$

Amidines have a pK_a only slightly higher than the primary amines from which they are derived,[8] so that the net charge and, more importantly, the overall conformation of a protein is not much perturbed, even by extensive amidination.[9] This feature is important, because alkyl imidates, especially when reacted with proteins under physiological conditions, are inefficient reagents, due to the relatively high rate of hydrolysis as compared with aminolysis.[6,10] As a result, many amino groups of a protein treated with an alkyl imidate cross-linker are modified with reagent hydrolyzed at the second end. Therefore, on the average, many molecules of cross-linker must be added before a cross-link is effected. If there were a loss of charge on each amino group modified, this could result in a major structural perturbation of the protein, so that a cross-link subsequently formed would not sample the native structure. Further, the nonproductive modification of amino groups typically results in a relatively low cross-linking yield, due to the loss of amino groups available for participation in a productive cross-link.

The problem of cross-linking yield is a severe one. Yields adequate to definitively identify specific cross-linked products are essential. In general, only positive results of cross-linking are interpretable; negative results cannot be interpreted with confidence.[6] Futher, low yields restrict the experimenter to studies involving relatively abundant proteins, and many membrane proteins of interest, e.g., hormone receptors, are relatively rare. An approach that has successfully overcome this problem is to focus on the yield of the modification reaction. If a reagent were subject to a very low rate of hydrolysis as compared with its rate of reaction with

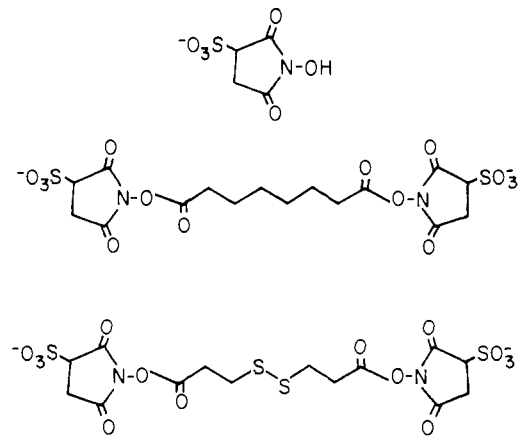

Figure 2. *N*-Hydroxysulfosuccinimide (A) and two cross-linking reagents in which this ligand is incorporated: bis(sulfo-*N*-succinimidyl) suberate (B) and 3,3′-dithiobis(sulfo-*N*-succinimidyl propionate) (C).[14]

nucleophilic groups in proteins, then very few molecules of cross-linker would be needed to produce a cross-link. A substantial reduction in the extent of modification necessary to yield a productive cross-link in turn leads to a lowered concern about perturbing the structure of the protein by the loss of charge due to the modification of any particular amino group. In the limit, one cross-linker would yield one cross-link.

Active esters appeared good candidates for high-yield, membrane-impermeant cross-linking reagents. Indeed, *N*-succinimidyl esters, which had been introduced by Anderson as intermediates for peptide synthesis,[11] had been successfully incorporated into protein cross-linking reagents.[12,13] However, while *N*-succinimidyl esters do hydrolyze slowly in aqueous solution,[12] their solubility characteristics make them unsuitable for use in membrane-impermeant reagents. Far from having the strongly hydrophilic character necessary, bifunctional *N*-succinimidyl esters generally must be dissolved in water-miscible organic solvents in order to be introduced into aqueous solution.[12,13]

Previous experience had shown that either a large, strongly hydrophilic ligand, e.g., a disaccharide, or a "hard" charge, i.e., an ionizable group with a pK_a very far from neutrality, is generally required to confer on a reagent membrane-impermeance. Further, in order to ensure impermeance, the final reagents should not closely resemble a natural substrate for transport by any of the many permeases that catalyze the translocation of hydrophilic metabolites across the membrane. These considerations led to the preparation of *N*-hydroxysulfosuccinimide as a hydrophilic leaving group for active esters.[14] This ligand was used to prepare bifunctional active esters of dicarboxylic acids for use as cross-linking reagents (Figure 2).[10,14]

Characteristics of Sulfo-*N*-succinimidyl Esters

Studies have been carried out to determine the relative rates of reaction of sulfo-*N*-succinimidyl esters with the nucleophilic groups in proteins and to determine

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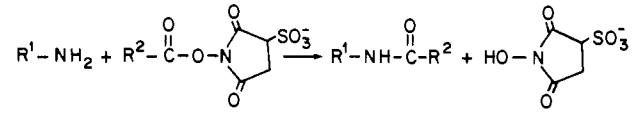
the kinetics of hydrolysis and aminolysis of these compounds, under conditions comparable to those used in protein modification reactions.[16] In aqueous buffer at pH 7.4 and room temperature, four nucleophilic groups were found to react with these compounds before the active esters hydrolyzed. The order of reactivity was found to be histidine imidazole > primary amino groups (either α or ϵ) $\gg$ cysteine thiolate $\approx$ tyrosine phenolate. The *N*-acylimidazole adduct with histidine is, however, a transient product, subject to hydrolysis or reaction with another nucleophile,[17] leaving amides formed by the acylation of primary amines as the major stable adduct:



The half-life for a model alkyl sulfo-*N*-succinimidyl ester in buffered aqueous solutions at 25 °C ranges from 1 h at pH 8 to >2 h at pH 7 in the absence of other nucleophiles.[16] This stability in aqueous solution, combined with very rapid reaction rates with primary amines, results in very high yields of adducts.

Stability to hydrolysis and strong hydrophilic character are important not only for cross-linking reagents but also for other applications. For example, addition of *N*-hydroxysulfosuccinimide to a water-soluble carbodiimide-mediated coupling reaction can enhance the yield by more than an order of magnitude.[18] This enhancement is presumably due to the formation of sulfo-*N*-succinimidyl esters in situ, which are more stable than the *O*-acylureas formed initially by the addition of the carbodiimide to carboxylate groups. Formation of the sulfo-*N*-succinimidyl esters has the effect of "rescuing" the carbodiimide-activated carboxylates for subsequent reaction with amines.

Kinetics of Cross-Linking

In order to evaluate the characteristics of a particular reactive groups for use in cross-linking reagents, one must consider the peculiar kinetics of cross-linking reactions. Each cross-linker undergoes two reactions. The reaction at the first end is, to a first approximation, kinetically second order—proportional to the concentration of cross-linker and to the effective concentration of the target nucleophile on the protein(s) to be cross-linked. However, once the first reaction has occurred, the second reactive group on the cross-linker is tethered to the protein surface, resulting in a high local concentration of the reactive group. If there is an appropriate nucleophile within the span of the reagent, the reaction will be greatly accelerated, relative to the rate of reaction at the first end.

The rate acceleration of the second reactive end of a cross-linker resulting from tethering the reagent to the protein surface has important consequences. For example, the number of different nucleophilic groups that react with significant yield is greater for the second end than it is for the first end, even when the two ends of the cross-linker are identical. The reason for this is straightforward. If a group reacts under certain conditions with nucleophiles X and Y, but the rate of reaction with X is 10^3 faster than with Y, the product mix for a monofunctional reagent with this group will have an insignificant yield of the Y adduct. However, a cross-linker tethered to a protein surface may be limited to a reaction volume that contains no X's, but one or more Y's. In this case, the increase in effective concentration of the reactive group can result in relatively efficient formation of the Y adduct.

The rate enhancement of reaction due to tethering the reagent to the protein surface can result in the preferential cross-linking of oligomeric complexes formed prior to the first reaction of the cross-linker, as compared with the cross-linking of components that randomly collide subsequent to the first reaction. However, while it is well established that in dilute solutions of proteins formation of intraoligomer cross-links is far more likely than interoligomer,[15] one must be cautious in experiments in which proteins in membranes are cross-linked. Protein concentration in a membrane is often very high, typically on the order of 50% (dry weight). Because proteins in membranes are restricted to diffusion in only two dimensions, the frequency of collisions can be very high. Cross-linking due to random collisions can usually be identified, because random collisions typically result in a heterogeneous product mix rather than a few discrete products. Cross-linking reactions are often analyzed by methods that separate the products of reaction by size, and collision complexes typically result in products with broad size distributions, whereas specific cross-linking yields products of discrete molecular weights.

Fortunately, many proteins in the plasma membranes of intact cells cannot diffuse freely. As discussed in the section on bifunctional spin-labeling reagents below, interactions of such membrane proteins with the cytoskeleton appear to cause severe restrictions on lateral and rotational diffusion. Cross-linking of such proteins in situ often yields discrete products that can be readily interpreted.

Cross-Linking Proteins in Membranes

Cross-linking studies of the anion-exchange channel in human erythrocytes provide a good example of the utility of membrane-impermeant reagents in studies of membrane-protein quaternary structure. The anion channel catalyzes the 1:1 exchange of Cl^- and HCO_3^- across the membrane and therefore is an important component in the transfer of CO_2, as HCO_3^-, from the distal tissues to the lungs.[19] A number of studies had suggested that the protein is oligomeric: Oxidation of membranes prepared from erythrocytes results in the formation of a disulfide bond formed by the oxidation of cysteinyl residues in two adjacent channel subunits.[4] Subsequent studies showed that the cysteinyl residues that contribute to the disulfide bond are located in the cytoplasmic domain of the protein.[20] Studies in which erythrocyte membrane proteins were solubilized with nondenaturing detergents generally supported a dimeric model,[21] but some cross-linking[5] and morphological[22]

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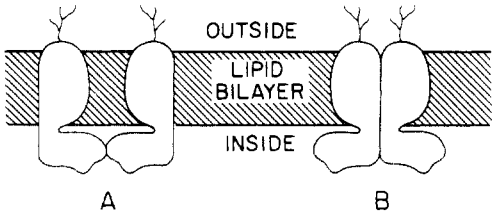

Figure 3. Schematic representation of two possible types of interaction between subunits of the anion-exchange channel in the erythrocyte membrane. "Outside" refers to the extracellular environment; "inside", to the cytoplasm.

studies suggested that a tetrameric model might be more appropriate. Proteolytic dissection of the structure of channel subunits showed that the protein could be cleaved into two structurally distinct fragments of roughly equal mass, a transmembrane domain that retains channel function and a soluble fragment corresponding to the cytoplasmic domain.[23]

Two questions concerning the quaternary structure of the anion channel that could be addressed by a membrane-impermeant cross-linking experiment were (1) what is the fundamental oligomeric unit? and (2) are the intersubunit associations solely between cytoplasmic domains (as schematically represented in Figure 3A) or are the transmembrane domains also juxtaposed (Figure 3B)? Treatment of intact erythrocytes with either of the cross-linking reagents shown in Figure 2, under conditions in which no leakage of reagent into the cells could be detected, resulted in the cross-linking of the large majority of anion channel subunits to dimers, with no detectable higher oligomers.[14,24] When cells were first treated with cross-linker to form the intersubunit cross-link in the extracytoplasmic portion of the transmembrane domain and then were lysed and the resulting membranes were oxidized to form the intersubunit disulfide bond in the cytoplasmic domain, the extracytoplasmic cross-link and the cytoplasmic disulfide bond were found to be between the same pair of subunits.[25]

These experiments support a dimer in which the transmembrane domains of two adjacent subunits are juxtaposed (Figure 3B) as the fundamental structural unit of the anion channel. The high yield of cross-linking places limits on oligomeric structures. If the channel were formed, for example, by four subunits arranged around a fourfold axis perpendicular to the membrane, the probability of cross-linking between any two adjacent subunits would be identical, so that under high-yield conditions, one would observe oligomers up to tetramer. It should be noted, however, that while these experiments exclude a symmetrical tetramer, they do not address the question of whether anion channel dimers might associate, perhaps by their cytoplasmic domains, to form higher order structures. However, subsequent studies using radiation inactivation analysis support the dimer as the functional unit of the anion channel.[26]

Thus far, I have focused on the quaternary structural information that can be obtained from a cross-linking experiment. When a protein is treated with a cross-linking reagent, *intra*subunit as well as *inter*subunit cross-linking is almost invariably effected. Since the dimensions of the cross-linking reagents and their adducts are known, mapping of the specific residues cross-linked would place severe restrictions on the folding of the polypeptide chain and thereby would provide important tertiary structural information. The use of cross-linking to determine the proximity of two residues in a folded protein is not a new concept; a number of cross-linking studies carried out 20–30 years ago on soluble proteins were directed to this purpose.[27]

Analysis of intrapolypeptide cross-linking for probing protein tertiary structure requires knowledge of the amino acid sequence of the protein. However, due to the advent of rapid indirect methods, based on sequencing complementary DNA to the messenger RNA coding for the protein of interest, there is a rapid and accelerating growth in the number of known sequences of membrane proteins of interest. In the case of the anion-exchange channel, such an indirect sequence analysis has been carried out for the murine protein,[28] and the human sequence is anticipated. From analysis of their sequence data, Kopito and Lodish have formulated a preliminary model for the way in which the polypeptide might fold, in which intramembranous and extramembranous segments are predicted.[28]

When the anion-exchange channel in intact erythrocytes is treated with bis(sulfo-*N*-succinimidyl) cross-linkers, such as those in Figure 2, intrasubunit as well as intersubunit cross-links are formed.[24,25] One of the intrasubunit sites is both functionally significant and readily monitored. There is a single site in the extracytoplasmic portion of the anion channel that is cleaved by chymotryptic treatment of intact erythrocytes, resulting in the cleavage of anion channel subunits into two large membrane-associated fragments.[29] The site of interest spans this chymotryptic cleavage site, so that anion channel subunits that have been cross-linked and chymotryptically cleaved do not separate into the two fragments when denatured.[24] Further, at low reagent concentrations, this site is kinetically preferred, and reaction at this site can be blocked by stilbene disulfonate inhibitors of anion transport.[25] Commercial radiolabeled bis(sulfo-*N*-succinimidyl) suberate (Amersham) should aid in detection of cross-linked peptides, and highly sensitive peptide sequencing methods[30] require that only small amounts of cross-linked peptides be purified. Iterative methods have been devised to aid in the unscrambling of the data resulting from the cosequencing of two cross-linked peptides[31] for use in aligning those sequences with the known sequence of the protein.[32] Experiments currently under way will test whether the promise of this method can be turned into practical experimental reality.

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(32) Sequence alignment can be carried out by using MATCH, a subroutine of PSQ, a program available from the Protein Identification Resource, National Biomedical Research Foundation, Georgetown University, Washington, DC 20007.

Cross-Linking of Membrane Proteins in Solution

As noted in the previous section, the situation can exist wherein the random collisions of proteins with their neighbors in the membrane could confuse the analysis of a cross-linking experiment. In such a case, it could be advantageous to solubilize the membrane proteins by the use of nondenaturing detergents[33] and carry out the cross-linking experiments in dilute solution. With this strategy, the membrane-impermeant character of the reagents is not per se an important property, but the strong hydrophilic character of the reagents is, and so too is their resistance to hydrolysis. Their hydrophilicity helps keep them in the bulk aqueous phase, rather than partitioning into the hydrophobic interior of the detergent micelles. As discussed in the section on kinetics, their resistance to hydrolysis helps to promote successful cross-linking at high dilutions of target protein.

An example of a solution cross-linking study of a membrane protein is a recent investigation into the effect of the hormone epidermal growth factor (EGF) on the oligomeric state of its receptor.[34] The binding of EGF to its plasma membrane receptor evokes a wide range of biochemical responses in the cell, and eventually, cell division.[35] The receptor has, as part of its structure, a catalytic domain with the enzymatic activity of a tyrosyl residue-specific protein kinase.[36] The binding of EGF to the receptor stimulates the activity of the kinase, and it is generally recognized that the EGF-induced stimulation of kinase activity is an important event in signal transduction; however, the mechanism by which binding of EGF results in kinase stimulation is not yet understood in detail.[37]

Morphological studies designed to follow the fate of EGF after binding to the cell surface showed that on binding EGF, receptors cluster, and the clusters coalesce in specialized areas of the cell surface known as coated pits.[35] The hormone–receptor complex is then removed from the cell surface by invagination of the coated pit and pinching off to form a vesicle inside the cell.[35] It has been suggested that the clustering observed at the morphological level might reflect oligomerization occurring at the molecular level and that EGF-induced receptor oligomerization might play an important role in kinase stimulation.[38] This concept is somewhat controversial,[37] and the study at hand is but one of several that have addressed it.

The A431 line of cultured cells, which was derived from a human epithelioid carcinoma, has been an important tool for studies of the EGF receptor.[39] This is due in large part because A431 cells overproduce EGF receptors, expressing approximately 2.4×10^6 receptors/cell,[40] which is about 50-fold normal. Cells were grown to a density of $(2–3) \times 10^7$ cells/flask and were solubilized with a buffer containing Triton X-100, a nondenaturing detergent.[34] This procedure results in a soluble extract containing membrane and cytoplasmic proteins, leaving behind extracellular matrix and most nuclear and cytoskeletal components. The calculated concentration of the receptor in this extract, assuming 100% solubilization, was $\approx 10^{-7}$ M. Samples of this extract were incubated with or without EGF and then were treated with 0–10 mM bis(sulfo-*N*-succinimidyl) suberate for 10 min. After the reaction was quenched, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a method that denatures proteins and separates them by molecular weight. The proteins were electrophoretically transferred from the gel to nitrocellulose sheets,[41] and the receptor was visualized by an immunostaining procedure. The result of this experiment is shown in Figure 4. EGF, which is itself monomeric in solution,[42] is seen to induce dimerization of the receptor. Similar cross-linking experiments with highly purified preparations of solubilized receptor suggest that EGF-induced receptor dimerization is an intrinsic property of the receptor that does not require any of the other cellular components present in the crude extract.[34]

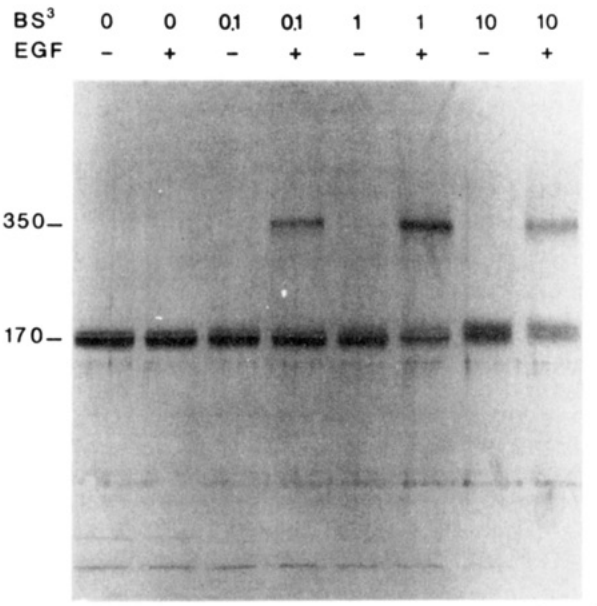

Figure 4. EGF-dependent cross-linking of its receptor to dimers.[34] Key: BS^3, bis(sulfo-*N*-succinimidyl) suberate (number to the right of BS^3 indicates concentration in mM); EGF, epidermal growth factor; +/−, presence or absence of 1 μM EGF in the reaction mixture; 170, position of migration of the 170-kDa monomeric receptor; 350, position of migration of the 350-kDa receptor dimer. (See text for details.)

In this experiment, solubilization of the receptor from the membrane was critical to demonstrate the specificity of EGF-induced receptor dimerization. The concentration of receptors in the membranes of A431 cells has been calculated to be $\approx 10^{-4}$ M,[37] and receptors in the membrane are restricted to two-dimensional diffu-

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sion, so that intermolecular collisions are much more likely to occur with the correct orientation than is the case for collisions between molecules in solution. Further, as noted above, EGF had been shown to induce clustering of receptors into small areas of the membrane, greatly increasing their local concentration. Solubilization of the receptors, with the large dilution that occurs as a result, yields conditions under which the specificity of dimer induction is much clearer.

Bifunctional Spin-Labeling Reagents as Probes of Dynamic Associations of Membrane Proteins

In addition to their roles in transmembrane processes such as transport and hormonal signal transduction, many membrane proteins function as points of connection for the membrane with the fibrous, polymeric structures in the cytoplasm collectively known as the cytoskeleton or membrane skeleton. The human erythrocyte provides a well-studied model.[43] In the erythrocyte, the anion-exchange channel provides a major point of attachment of the cytoskeleton through a specific linker protein, ankyrin.[19] There is a good support for the hypothesis that the interaction between the anion channel and the cytoskeleton is dynamic.[44] In circulation erythrocytes are highly elastic. It is the property of elastic deformation that allows erythrocytes, which at rest are biconcave disks of ≈8-μm diameter, to traverse capillaries of 3–4-μm diameter. This elasticity can be interpreted as a macroscopic reflection of dynamic interactions among cytoskeletal and membrane proteins. There is also ample evidence that this normal dynamic state requires energy in the form of ATP:[44] For example, on storage, under conditions that lead to the depletion of the intracellular ATP pool, erythrocytes exhibit significantly decreased deformability and they undergo changes in shape. In order to be able to test aspects of this hypothesis relating to the interactions between the cytoskeleton and the anion channel, it would be useful to measure the rotational mobility of the channel in the membranes of intact cells as an indicator of interactions between the channel and the cytoskeleton. Together with Dr. A. H. Beth and his co-workers, we have developed EPR spectroscopic methods for making such measurements.[45–48]

The use of EPR methods for measuring the rotational diffusion of proteins has been limited by the experimenter's ability to tightly couple a paramagnetic ligand, usually a nitroxide spin label, to the protein of interest. In the sense used here, "tightly couple" refers to the overall motion of the protein, so that a ligand covalently linked to a protein but in such a way that local rotational motion is allowed would not be tightly coupled, as the motion of the ligand would not report the overall rate of tumbling of the protein. It was suggestion of Dr. Beth, who has played a central role in the development of EPR spectroscopic techniques for measuring slow

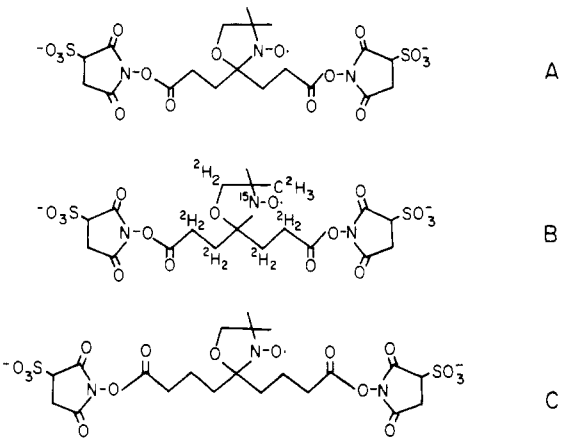

Figure 5. Three bifunctional spin-labeling reagents: bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate,[45] bis(sulfo-*N*-succinimidyl) [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelate (^{15}N,$^{2}H_{16}$-BSSDP),[47] and bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate.[48] The isotopic substitutions in ^{15}N,$^{2}H_{16}$-BSSDP result in significant spectral enhancements.[47,49]

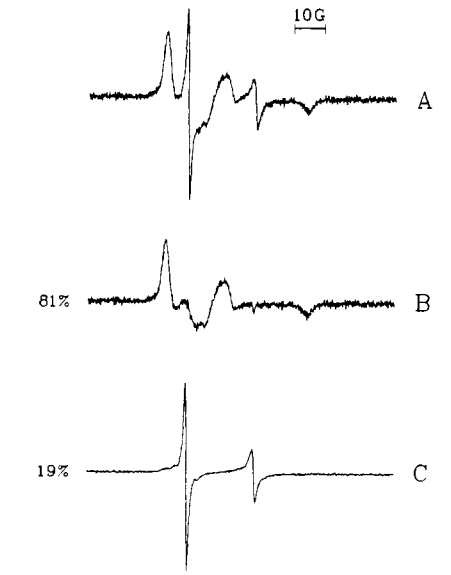

Figure 6. Linear EPR spectrum of erythrocytes labeled with ^{15}N,$^{2}H_{16}$-BSSDP and its component spectra. (Modified from ref 47. See text for details.)

rotational motion,[49] that a general solution to this problem might be reactively bifunctional spin labels, which would more tightly couple to the rotational diffusion of a labeled protein by virtue of their bidentate linkage. This idea[50] led to the preparation of a small family of bifunctional spin-labeling reagents related to bis(sulfo-*N*-succinimidyl ester) cross-linkers (Figure 5).

The reagents shown in Figure 5 react with the anion-exchange channel similarly to the non-spin-label cross-linkers shown in Figure 2, so that at low reagent concentrations, reaction is favored with the intrasubunit site that spans the extracellular chymotryptic cleavage site and overlaps the stilbenedisulfonate site.[45–47] Under appropriate conditions, this single site can be specifically modified when *intact cells* are treated with reagent.[45–47] A linear EPR spectrum of a suspension of erythrocyte treated under such conditions with

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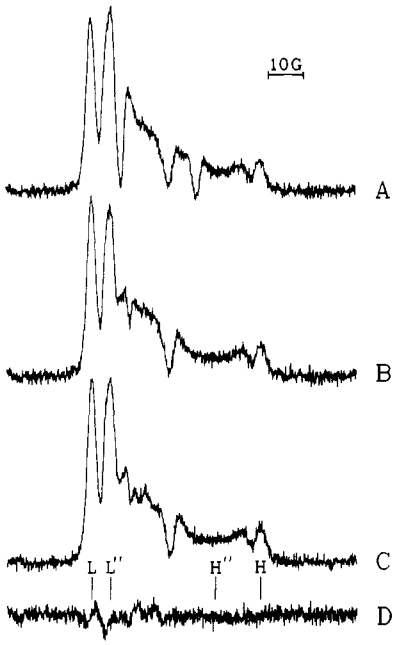

Figure 7. Saturation transfer spectra of erythrocytes labeled with $^{15}N,^{2}H_{16}$-BSSDP. (Modified from ref 47. See text for details.)

$^{15}N,^{2}H_{16}$-BSSDP is shown in Figure 6A. The spectrum consists of two components. The partially immobilized component (Figure 6C) is from labeled lipids, while the strongly immobilized signal (Figure 6B) is from label bound to the intrasubunit site of the anion channel.[45-47] The strongly immobilized signal is at the linear EPR no-motion limit, indicating an effective rotational correlation time, $\tau_r \geq 1$ μs. The absence of dipolar interactions between labels on adjacent subunits of an anion channel dimer suggests that the nitroxide moieties are separated by a *minimum* of 16 Å,[47] suggesting a surprisingly large separation between the two extracytoplasmic inhibitor sites present in each channel dimer.

Since the τ_r of the channels is too large to be measured by linear EPR techniques, saturation transfer EPR methods[49] are required. A saturation transfer spectrum of $^{15}N,^{2}H_{16}$-BSSDP-labeled intact erythrocytes is shown in Figure 7A. Figure 7B is the saturation transfer spectrum of labeled anion channels, resulting from digital subtraction of the labeled lipid component of the spectrum in Figure 7A. While an accurate assessment of τ_r using a computational modeling approach requires orientation data,[49] which we are only now in a position to obtain, useful comparative analyses can be made. Figure 7C is the saturation transfer spectrum of $^{15}N,^{2}H_{16}$-BSSDP-labeled anion channels in intact erythrocytes that have been cross-linked to covalent dimers with bis(sulfo-*N*-succinimidyl) suberate. Figure 7D is the difference spectrum, 7B – 7C. The observation that the difference spectrum is virtually flat suggests that the rotational diffusion of the cross-linked and non-cross-linked channels is essentially identical. This observation is consistent with the idea that the channel is a stable dimer.

The next stage of these experiments will focus on how the level of the intracellular ATP pool affects the populations of anion channels bound or not bound to the cytoskeleton. Shifts in the two populations are predicted to have measurable effects on the observed τ_r of the channels, so the effects of ATP levels on the dynamic interactions of the channels with the cytoskeleton can hopefully be monitored.

In these experiments we have made use of the inherent affinity of the anion channel for the dianionic reagents. How might these methods be more generally applied to membrane protein dynamics? One promising approach is to use the bifunctional spin labels to prepare derivatives of polypeptide hormones. To be useful, the derivative must exhibit tight motional coupling between label and hormone, and the modification must not interfere with normal binding or biological activity. Recently, we prepared such a derivative of epidermal growth factor with both normal isotope and $^{15}N,^{2}H_{16}$-BSSDP.[51] In very preliminary experiments, incubation of one of these derivatives with membranes from A431 cells resulted in an EPR spectrum in which bound and free hormone could be resolved. In principle, hormone derivatives such as this will be useful for studies of hormone binding and dissociation, as well as studies of rotational diffusion of the occupied receptor in the membrane.

Conclusions

The development of high-yield, membrane-impermeant cross-linking reagents has given rise to new experimental approaches to understanding the structure and function of membrane proteins. With various of these reagents, time-average tertiary and quaternary structures can be probed by cross-linking, and the dynamic interactions among membrane proteins and supramolecular cellular structures can be probed spectroscopically.

The author gratefully acknowledges support from the National Institutes of Health: Grants DK31880, DK25489, and CA43720. The author thanks Drs. A. H. Beth, J. Jentoft, T. Macdonald, and J. E. Nieves for helpful comments and S. Heaver for assistance in the preparation of the manuscript.

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