matrix-bound enzyme is controversial (Cho & Swaisgood, 1972, 1974; Chan & Mosbach, 1976; Ashmarina et al., 1981). Whether the matrix in this case "mimics" the subunit interface in the native quaternary structure or whether flexibility of the chain molecules forming the matrix is involved needs to be clarified.

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N-Hydroxysulfosuccinimide Active Esters: Bis(*N*-hydroxysulfosuccinimide) Esters of Two Dicarboxylic Acids Are Hydrophilic, Membrane-Impermeant, Protein Cross-Linkers†

James V. Staros

ABSTRACT: We have synthesized and characterized *N*-hydroxysulfosuccinimide, a new hydrophilic ligand for the preparation of active esters. We have incorporated this ligand into two new protein cross-linking reagents, 3,3′-dithiobis(sulfosuccinimidyl propionate) and bis(sulfosuccinimidyl) suberate. In experiments with rabbit muscle aldolase, it is demonstrated that both of these reagents are highly efficient protein cross-linkers at physiological pH and that 3,3′-dithiobis(sulfosuccinimidyl propionate) is quantitatively cleavable by reduction under mild conditions. In experiments with intact human erythrocytes and erythrocyte membranes, it is shown that both reagents are membrane impermeant and that when erythrocytes are treated with either reagent, both cross-link subunits of the anion channel (band 3) to covalent dimers at the extracytoplasmic membrane face.

We have recently demonstrated the utility of a membrane-impermeant, cleavable cross-linker for probing near-est-neighbor relationships of membrane protein subunits at one face of a biological membrane (Staros et al., 1981). The reagent that we introduced for this purpose, diisethionyl 3,3′-dithiobis(propionimidate), is a bis(alkylimidate) and is a membrane-impermeant analogue of dimethyl 3,3′-dithiobis(propionimidate), introduced by Wang & Richards (1974a,b, 1975).

As protein modification reagents, alkylimidates have the distinct advantage that the product amidines are charged at physiological pH like the primary amines from which they are

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derived (Wold, 1972; Peters & Richards, 1977). This retention of charge is the apparent explanation for the observation that even extensive amidination leads to only minimal perturbations of overall protein structure (Wofsy & Singer, 1963; Hunter & Ludwig, 1972). However, under conditions suitable for protein cross-linking, alkylimidates are subject to hydrolysis at rates which are significant when compared with those for amidine formation (Browne & Kent, 1975; Peters & Richards, 1977). In cross-linking experiments, hydrolysis can markedly reduce yields. Thus, in cross-linking experiments in which erythrocyte membranes were reacted with dimethyl adipimidate, Niehaus & Wold (1970) showed that only 20% of the modified lysines participated in cross-links; the other 80% were modified with half-hydrolyzed reagent. Half-hydrolysis to this extent can, in principle, be of great importance when only a limited number of lysyl residues are appropriately positioned for cross-linking within a protein oligomer.

One approach to a solution to the problem of hydrolysis has been the development of cross-linkers that are bis(*N*-hydroxysuccinimide) esters of dicarboxylic acids (Lomant & Fairbanks, 1976; Pilch & Czech, 1979). Such reagents have been shown to undergo hydrolysis very slowly as compared with aminolysis (Lomant & Fairbanks, 1976) and thus have the potential of significantly higher cross-linking yields than do analogous bis(alkylimidates). However, these reagents are very hydrophobic; they typically must be dissolved in a water-miscible organic solvent in order to be introduced into aqueous solutions (Lomant & Fairbanks, 1976; Pilch & Czech, 1979). Here are described the syntheses of 1-hydroxy-3-sulfo-2,5-pyrrolidinedione (*N*-hydroxysulfosuccinimide), a sulfonated analogue of *N*-hydroxysuccinimide, and of cleavable and noncleavable cross-linkers prepared by coupling this reagent to dicarboxylic acids. These cross-linkers are demonstrated to be readily water-soluble, membrane-impermeant probes.

Experimental Procedures

All chemicals were of ACS certified grade, or of a comparable or higher grade. Water was deionized and then glass distilled. Fourier transform, natural abundance ^{13}C NMR spectra were obtained on a JEOL FX90Q spectrometer. ^{1}H NMR spectra were obtained on a JEOL JNM-MH-100 spectrometer. IR spectra were obtained on a Perkin-Elmer 337 spectrophotometer. Ion-pair reverse-phase HPLC[1] was carried out in an isocratic system consisting of a Constametric III pump (Laboratory Data Control), a manual injector with 20-μL sample loop (Rheodyne, Model 7125), a C_{18} column (Alltech, no. 600-RP), and a UA-5 detector (ISCO, Inc.) (set at 254 nm) fitted with a micro flow cell having a 2-mm light path and a 4-μL illuminated volume. The mobile phase was 60:40 (system 1) or 50:50 (system 2) 10 mM tetrabutylammonium formate (pH 4.0)–methanol. TLC was carried out on 0.20-mm silica gel with fluorescent indicator on Al backing (EM Laboratories). Plates were developed in 5:2:3 1-butanol–acetic acid–water. One- and two-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis and preparation of samples for electrophoresis were carried out as previously described (Staros et al., 1981).

N-Hydroxysulfosuccinimide Sodium Salt (1-Hydroxy-3-sulfo-2,5-pyrrolidinedione Sodium Salt). To a solution of *N*-hydroxymaleimide (Fluka) (1.45 g, 12.8 mmol) in absolute ethanol under N_2 was added an aqueous solution of $Na_2S_2O_5$ (1.22 g, 6.4 mmol). The reaction mixture was stirred for 2 h at room temperature, under N_2, and then was opened to the atmosphere. After the solvent was stripped in a rotary evaporator (bath temperature ~40 °C), the product, a thick yellow oil, was dissolved in 50 mL of H_2O, filtered, and lyophilized. The resulting light yellow solid was triturated with anhydrous ether, and then the product, an off-white powder, was recovered by filtration; yield 2.66 g (96%). The product gives a single spot on TLC (R_f 0.32). Ion-pair reverse-phase HPLC (system 1) gives a single peak accounting for 97% of the total area of the chromatogram (determined gravimetrically). This product was used for further synthesis, but a small quantity was recrystallized from aqueous ethanol for analytical purposes. The crystals do not melt (to 300 °C) but slowly decompose at ~250 °C and above. Anal. Calcd for $C_4H_4NNaO_6S$: C, 22.12; H, 1.86; N, 6.45; Na, 10.59; O, 44.21; S, 14.76. Found: C, 22.02; H, 2.04; N, 6.29; Na, 10.58; O, 44.36; S, 14.69 (Galbraith Laboratories).

Carboxylic Acid N-Hydroxysulfosuccinimide Active Esters. $HOSu(SO_3)Na$ (2.0 mmol), the carboxylic acid to be derivatized (2.0 mmol, i.e., 1.0 mmol of a dicarboxylic acid), and DCC (2.2 mmol) were dissolved in 5.0 mL of dry dimethylformamide, and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then cooled to 3 °C and stirred 2–3 h longer. The precipitated dicyclohexylurea was removed by filtration and was washed with a small quantity of dry dimethylformamide. The product was then precipitated from solution by addition of ~20 volumes of ethyl acetate, recovered by filtration, and stored in a vacuum desiccator. By this method, Na_2BS^3 was prepared at 68% yield and Na_2DTSSP at 65% yield (calculated assuming a pure, anhydrous product). The IR spectra of both compounds exhibit relatively strong and broad sulfonate resonances with maxima at 1220 and 1045 cm^{-1} which are predominant characteristics of the spectrum of the parent $HOSu(SO_3)Na$. When samples of these compounds were hydrolyzed to completion, $HOSu(SO_3)$ was quantitatively released, as analyzed by HPLC (system 2).

Cross-Linking of Rabbit Muscle Aldolase. A crystalline suspension of rabbit muscle aldolase in 2.5 M $(NH_4)_2SO_4$ (Sigma type IV) was extensively dialyzed against 50 mM sodium phosphate, pH 7.4. The final concentration of aldolase was determined by absorbance at 280 nm (Donovan, 1964). Equal aliquots of this solution were diluted with 50 mM sodium phosphate, pH 7.4, and were treated with various concentrations of DTSSP or BS^3. (The cross-linkers were prepared immediately before use as 10 mM stock solutions in 50 mM sodium phosphate, pH 7.4.) After the solutions were incubated for 30 min at room temperature, the reactions were quenched by addition of $^1/_6$ volume of 50 mM ethanolamine, 20 mM *N*-ethylmaleimide,[2] and 50 mM sodium phosphate, pH 7.4. Each reaction was split into two aliquots, treated with solubilizing solution with or without dithiothreitol as described (Staros et al., 1981), incubated at 50 °C for 3 min, and then stored at −65 °C.

Cross-Linking of Intact Erythrocytes. Erythrocytes from normal human donors were prepared from whole blood stored

[1] Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; $HOSu(SO_3)$, *N*-hydroxysulfosuccinimide (1-hydroxy-3-sulfo-2,5-pyrrolidinedione); $HOSu(SO_3)Na$, $HOSu(SO_3)$ sodium salt; BS^3, bis(sulfosuccinimidyl) suberate; Na_2BS^3, BS^3 disodium salt; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); Na_2DTSSP, DTSSP disodium salt; DCC, dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DIDIT, diisethionyl 3,3′-dithiobis(propionimidate).

[2] *N*-Ethylmaleimide was added to quench buffers to alkylate free thiols and thereby inhibit thiol–disulfide exchange (Wang & Richards, 1974b; Staros et al., 1981).

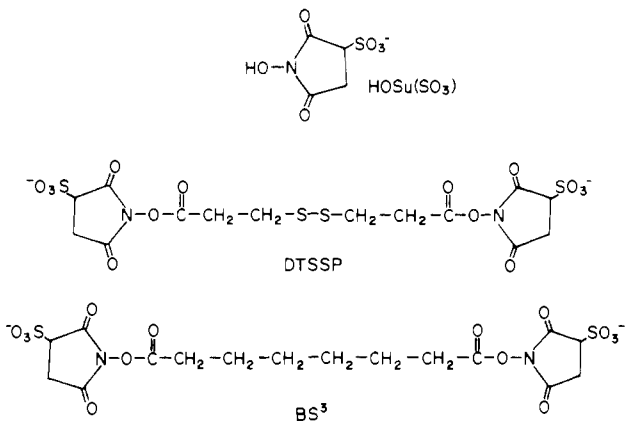

FIGURE 1: Structures of *N*-hydroxysulfosuccinimide [HOSu(SO_3)], 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), and bis(sulfosuccinimidyl) suberate (BS^3).

in acid–citrate–dextrose (≤1 week from drawing) as previously described (Staros & Richards, 1974). Erythrocytes were washed once in 106 mM sodium phosphate, pH 7.4, and then the packed cells were resuspended in an equal volume of the same buffer. Cells suspended in this buffer were reacted with various concentrations of DTSSP or BS^3 for 30 min at room temperature. The reaction mixtures were agitated gently and constantly during the reaction time. The reaction was then quenched by the addition of ~10 volumes of 0.15 M NaCl and 25 mM Tris, pH 7.4. The cells were pelleted by centrifugation, and the absorbance at 550 nm was measured to monitor lysis during the reaction. By this measure, lysis was <0.5% when compared to the supernatant of a control lysate. After being washed once more in the quench buffer, the cells were lysed at a hemolytic ratio of 80 in 0.1 mM Na_2EDTA and 20 mM *N*-ethylmaleimide,[2] adjusted to pH 7.4 with Tris (lysis buffer). The resulting ghosts were washed twice in 15 mM NaCl, 1.7 mM Tris, and 0.1 mM Na_2EDTA, pH 7.4 (ghost wash buffer), resuspended in the same buffer, treated with solubilizing solution without dithiothreitol, incubated at 37 °C for 20 min, and then frozen at −65 °C until needed.

Cross-Linking of Erythrocyte Ghosts. Washed, packed erythrocytes were lysed in 40 volumes of ice-cold lysis buffer, and the resulting ghosts were washed twice in 0.1 mM Na_2-EDTA and 10 mM sodium phosphate, pH 7.4. Aliquots (0.2 mL) of the ghost pellet were resuspended in an equal volume of the same buffer and were treated with various concentrations of DTSSP. The reactions were allowed to proceed for 30 min at room temperature with constant gentle agitation and were then quenched by addition of ~20 volumes of 5.0 mM Tris and 5.0 mM sodium phosphate, pH 7.4. After centrifugation, the ghosts were resuspended in the same buffer, then solubilized with solubilizing solution without dithiothreitol, incubated for 3 min at 50 °C, and stored at −65 °C until needed.

Results

Synthesis of HOSu(SO_3)Na (Figure 1) was accomplished by sulfitolysis (Micich et al., 1975) of *N*-hydroxymaleimide under an inert atmosphere. HOSu(SO_3) was characterized by consistent elemental analysis and IR spectrum and by a thorough NMR study. The 100-Mz ^1H NMR spectrum of HOSu(SO_3)Na in deuterated dimethyl sulfoxide contains a multiplet (δ 3.05, 2 H)[3] assigned to the methylene protons and a doublet of doublets (δ 4.0, J = 9.0, 4.5 Hz, 1 H) assigned to the proton α to the sulfonate. The spectrum of HOSu(SO_3) in D_2O initially includes the same two resonances (δ 3.35, 2 H; δ 4.5, J = 9.0, 4.5 Hz, 1 H). After several days of incubation in D_2O, the signal from the proton α to the sulfonate disappears (corresponding to H–D exchange of that proton), and the methylene protons appear as two doublets with geminal splitting (δ 3.0, 3.27, J_{gem} = 19 Hz). Likewise, the initial ^{13}C NMR spectrum of HOSu(SO_3) in D_2O contains the following resonances: 27.7 ppm, assigned to the methylene C; 54.4 ppm, assigned to the C bearing the sulfonate; 167.3 ppm, assigned to the carboxyl C β to the sulfonate; 171.5 ppm, assigned to the remaining carbonyl C. After incubation in D_2O as above, the sharp resonance at 54.4 ppm is replaced by a weak triplet, confirming the assignment of that resonance to the C bearing the sulfonate.

Na_2BS^3 and Na_2DTSSP were prepared by coupling HOSu(SO_3)Na to suberic acid and to 3,3′-dithiodipropionic acid, respectively, by the DCC method (Anderson et al., 1964). These compounds were characterized by consistent IR and ^1H NMR spectra and by the observation that hydrolysis yields free HOSu(SO_3).

Rabbit muscle aldolase is readily cross-linked to oligomers by either DTSSP (Figure 2A) or BS^3 (Figure 2C). Reduction of the disulfide bonds in DTSSP-modified aldolase cleanly cleaves the cross-links, yielding aldolase monomers on Na-DodSO_4–polyacrylamide gel electrophoresis (Figure 2B). That the disulfide bond in the DTSSP moiety is the point of cleavage is demonstrated by a comparison of parts B and C of Figure 2. While reduction quantitatively yields monomers in the DTSSP cross-linked samples, reduction has no effect on the BS^3 cross-linked samples.

Unsealed erythrocyte ghosts were cross-linked with DTSSP, and the ghosts were dissolved with NaDodSO_4 and subjected to the two-dimensional electrophoresis procedure devised by Wang & Richards (1974b). In this procedure, the first dimension is run under nonreducing conditions, and the second dimension is run under reducing conditions. The results of such an experiment are shown in Figure 3. The pattern obtained is comparable to that resulting from the use of other cleavable cross-linkers in comparable experiments (e.g., Wang & Richards, 1974b; Staros et al., 1981). Spectrin (Marchesi & Steers, 1968) (bands 1 and 2) is the most readily cross-linked protein; a large proportion of the spectrin is cross-linked in this experiment to very large oligomers which cannot appreciably enter the first-dimension gel. The anion channel (Rothstein et al., 1976) (band 3) is cross-linked to dimers. Actin (Tilney & Detmers, 1975) (band 5) is cross-linked to dimers, and glyceraldehyde-3-phosphate dehydrogenase (Kant & Steck, 1973) (band 6) is cross-linked to dimers (and in the original gel trimers and tetramers are also visible). A large hetero oligomer is formed between spectrin and many of the other membrane components, resulting in the vertical streak at the left-hand margin of the gel.

In sharp contrast to the extensive cross-linking which results from DTSSP treatment of ghosts, DTSSP cross-linking of intact erythrocytes yields only one major product, identified as a dimer of the anion channel, band 3 (Figure 4). Likewise, when intact erythrocytes are treated with increasing concentrations of the noncleavable cross-linker BS^3, an increasing proportion of band 3 disappears from the one-dimensional gel electrophoresis pattern, and simultaneously, a new component at M_r 1.9×10^5 increases, suggesting that band 3 is cross-linked to dimers in intact erythrocytes by BS^3 (Figure 5). This is the only significant change in the NaDodSO_4–polyacryl-

[3] All chemical shifts cited were measured downfield of a hexamethyldisilazane external standard.

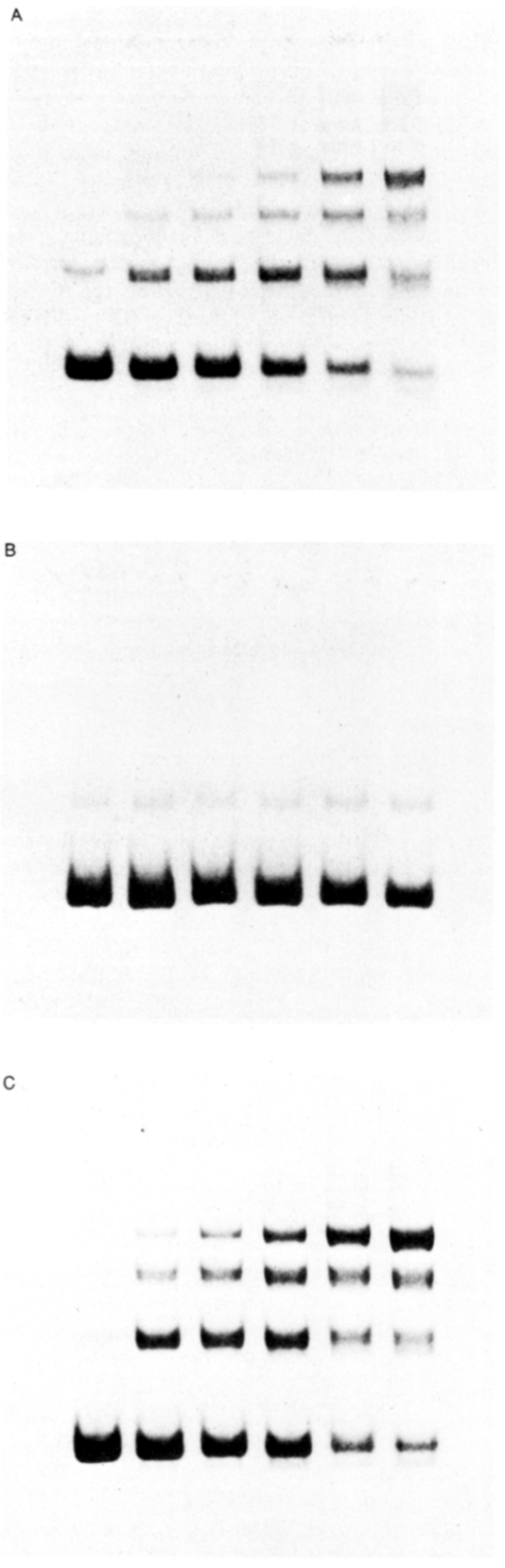

FIGURE 2: Cross-linking of rabbit muscle aldolase. (A) Aldolase (1.0 mg/mL) cross-linked with DTSSP at reagent concentrations of 0, 0.05, 0.10, 0.20, 0.50, and 1.0 mM, solubilized, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis under nonreducing conditions. (B) Aliquots of the same samples as in (A) but solubilized and subjected to electrophoresis under reducing conditions. (C) Aldolase (1.0 mg/mL) cross-linked as in (A) but with BS^3, then treated with solubilizing solution with dithiothreitol, and subjected to electrophoresis under reducing conditions as in (B). The photographs are of the dried, Coomassie blue G-250 stained gels.

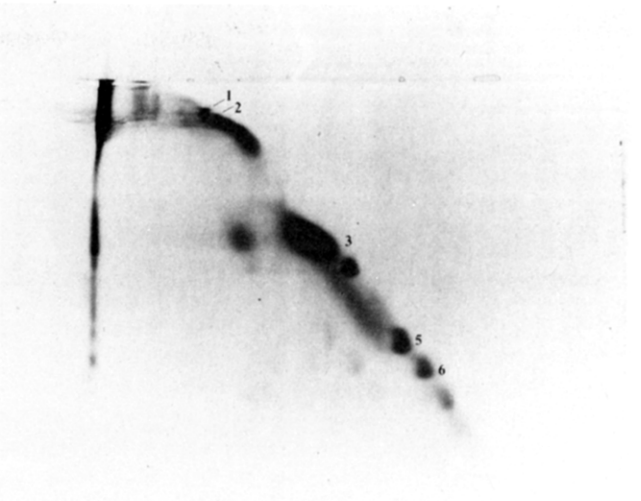

FIGURE 3: Erythrocyte ghosts cross-linked with DTSSP. Erythrocyte ghosts were cross-linked with DTSSP (3 mM), solubilized, and subjected to two-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis, as described in the text. The positions of bands 1, 2, 3, 5, and 6 (nomenclature of Fairbanks et al., 1971) are indicated. The photograph is of the wet gel which had been stained with Coomassie blue G-250. Spots to the left of the diagonal of un-cross-linked proteins are derived from oligomers cross-linked with DTSSP.

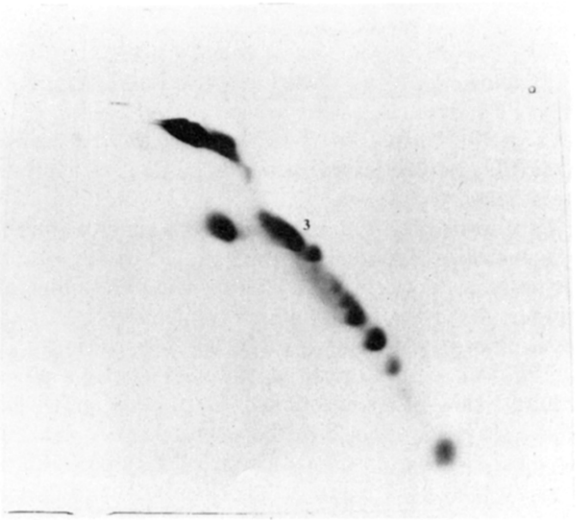

FIGURE 4: Intact erythrocytes cross-linked with DTSSP. Membranes isolated from intact erythrocytes which had been reacted with DTSSP (5 mM) were solubilized and subjected to the same two-dimensional electrophoresis procedure as was the sample in Figure 3. The position of un-cross-linked band 3 on the diagonal is indicated. The spot to the left of band 3 and in horizontal register with it arose from the DTSSP-mediated cross-linking of band 3 to dimers at the extracytoplasmic membrane face. The photograph is of the wet, stained gel, as in Figure 3.

amide gel pattern of erythrocyte membranes associated with BS^3 treatment of intact erythrocytes.

Discussion

Membrane-impermeant protein cross-linkers can provide three-dimensional information concerning the intersubunit contacts of membrane protein oligomers (Staros et al., 1981; Staros, 1982). Using these probes, one can experimentally pose not only the question of which subunits are adjacent to which others but also the question of at which face of the membrane such interactions occur.

In an effort to produce a reagent which would have the membrane-impermeant character of DIDIT (Staros et al., 1981) but which would cross-link proteins in higher yield and

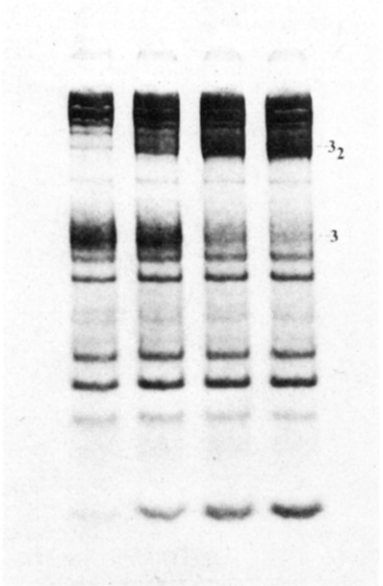

FIGURE 5: Intact erythrocytes cross-linked with BS^3. Membranes from erythrocytes which had been treated with 0, 1, 3, or 5 mM BS^3 were solubilized and subjected to one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions as described in the text. The positions of band 3 and band 3 dimer (3_2) are indicated. The photograph is of the dried, stained gel, as in Figure 2.

at physiological pH, we turned to active esters. Our success in using a sulfonated leaving group in DIDIT led to the synthesis of the sulfonated *N*-hydroxysuccinimide derivative $HOSu(SO_3)$ as the leaving group for a class of hydrophilic active esters.

That BS^3 and DTSSP are efficient protein cross-linkers at physiological pH is clearly demonstrated by the results of cross-linking aldolase with these reagents. The results shown in Figure 2 show a dramatic improvement in yield over the cross-linking of aldolase with DIDIT (Figure 2 in Staros et al., 1981) or with other bis(alkylimidates) (e.g., Wang & Richards, 1974a; Davies & Stark, 1970). Not only does one see the sharp cutoff in yield between tetramer and higher oligomers (Figure 2A,C) indicative of a native tetrameric structure (Davies & Stark, 1970) but also one can see with increasing concentration of DTSSP or BS^3 the buildup first of covalent dimer, and then of covalent tetramer, without the intermediate dominance of trimer, suggesting a dimer of dimers structure for the native tetramer. That the disulfide bond in DTSSP can be quantitatively cleaved by reduction is also demonstrated in Figure 2.

That DTSSP is a membrane-impermeant, cleavable cross-linker is demonstrated by a comparison of Figures 3 and 4. When leaky ghosts are reacted (Figure 3), cytoplasmic face components such as spectrin and glyceraldehyde-3-phosphate dehydrogenase are readily cross-linked. As with DIDIT (Staros et al., 1981) and with the membrane-permeant cross-linker dimethyl 3,3′-dithiobis(propionimidate) (Wang & Richards, 1974b, 1975), spectrin is the most readily cross-linked of the proteins associated with the membrane. However, when intact erythrocytes are cross-linked with DTSSP (Figure 4), the only significant product in the Coomassie blue stained pattern is the dimer of band 3. This is the same qualitative result as was obtained with DIDIT (Staros et al., 1981), but quantitatively, DTSSP produces covalent dimers of band 3 much more efficiently than does DIDIT. [Compare Figure 4 with Figure 4 in Staros et al. (1981).] Likewise, when intact erythrocytes are treated with BS^3, only band 3 is seen to be cross-linked (Figure 5). Since this protein [see Steck (1978) and Knauf (1979) for reviews] is well-known to have a transmembrane orientation and to be accessible to the extracellular milieu, we interpret the above results to indicate that DTSSP and BS^3 are impermeant to the erythrocyte membrane, at least under the conditions tested.

While DTSSP and BS^3 give a higher cross-linking yield than does DIDIT, it must be recognized that there is a trade-off for this higher yield (Staros, 1982). Amidines which are produced by reaction of alkyl imidates with primary amino groups in proteins retain the positive charge of the parent amino groups at neutral pH. However, BS^3 and DTSSP, by analogy with *N*-hydroxysuccinimide active esters, presumably acylate amino groups and thereby abolish their charge. Such an abolition of a charged group near the interface of the hydrophobic domain of a membrane with the aqueous milieu might lead to structural changes in the modified protein which could be sampled by subsequent reaction. We therefore suggest that DTSSP and DIDIT are complementary reagents, one maximizing yield of cross-linking and the other retention of native charge.

The successful use of $HOSu(SO_3)$ in membrane-impermeant cross-linkers suggests that it might be useful for producing active esters for other applications requiring aminoacylations in aqueous solutions.

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Orthogonal Packing of β-Pleated Sheets in Proteins†

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ABSTRACT: Two classes of β-sheet to β-sheet packing can be distinguished in globular proteins. Both classes have β sheets with the usual right-handed twist packed face to face. In *orthogonal β-sheet packings*, the strand directions of the different β sheets are 90° to each other. Twisted β sheets in this orientation have anticomplementary surfaces: one pair of diagonally opposite corners in the β sheets is very close, and the other pairs of corners splay apart. At the close corners, the β sheets are usually covalently connected: a strand that is part of one β sheet turns through a right-handed bend to become part of the second β sheet. The bend may occur at a β bulge, or over a stretch of residues with a characteristic conformation, forming what we call a *β bend*. Contacts between the β sheets occur along the diagonal joining the close corners. They involve about one-fourth of the β-sheet residues, and two-thirds of them are Val, Ile, or Leu. Elsewhere, the space between the β sheets is filled by side chains from other parts of the protein, often α helices placed at the splayed corners. Examples of orthogonal β-sheet packing are found in alcohol dehydrogenase, the acid proteases, the trypsin family, papain, staphylococcal nuclease, and thermolysin. *In aligned β-sheet packings*, the angle between the strand directions of the packed β sheets is ~−30°. In this orientation, the twisted β-sheet surfaces are complementary. The principles governing this class of β-sheet packings have been described previously. Here we discuss the differences and similarities of the aligned and orthogonal packing classes.

In proteins, the polypeptide chain folds into α helices and β-pleated sheets, which close-pack to form the three-dimensional structure. It has been shown that, underlying the great variety of protein structures, there are *gerneral principles* that govern how α helices pack onto other α helices and onto β-pleated sheets (Crick, 1953; Chothia et al., 1977, 1981; Richmond & Richards, 1978; Efimov, 1977, 1979; Cohen et al., 1980, 1981; Janin & Chothia, 1980). We discuss here the principles that govern the packing together of β-pleated sheets.

Proteins whose secondary structure consists mostly of β-pleated sheets have a number of common features. They are layer structures, usually bilayers or "sandwiches". Each layer is a β sheet with a right-handed twist and forms face to face contacts with other layers (Figure 1). The relative orientation of the β sheets in contact defines two classes of structures (Chothia et al., 1977). In one class, the mean direction of the β strands in one layer is at a small angle (about −30°) to that of the β strands in the other layer. An example of this class is prealbumin. In the second class, this angle is close to 90°. The packing of the β sheets in the first class will be referred to as *aligned β-sheet packing*, as opposed to the *orthogonal β-sheet packing* of the second class.

Detailed descriptions of aligned β-sheet packings have been published (Cohen et al., 1981; Chothia & Janin, 1981), and most of this paper will be devoted to an analysis of orthogonal β-sheet packings. We present a general model and compare it to what is actually observed in proteins that contain orthogonally packed β sheets. In addition, we define a peculiar conformation of the polypeptide chain, *the β bend*, which enables β strands to bend by 90° without disturbing their H-bond pattern.

Experimental Procedures

We have used computer graphics and numerical calculations to examine in detail the conformation and packing of the β-pleated sheets in five proteins whose structures have been determined by X-ray analysis: alcohol dehydrogenase, catalytic domain (Eklund et al., 1976); elastase, first domain (Sawyer et al., 1978); papain, second domain (Drenth et al., 1971); penicillopepsin, first domain (Hsu et al., 1977); staphylococcal nuclease (Arnone et al., 1971).

Elastase and penicillopepsin are taken as representatives of two large families of homologous structures: elastase for trypsin-like serine proteases and penicillopepsin for acid proteases. Residue numbering for elastase is based on its alignment with chymotrypsin and that for penicillopepsin on its alignment with pig pepsin.

A general model for orthogonal β-sheet packing is presented in Figure 2. This model will be useful in understanding the relation and coherence of the rather complex structural descriptions given in the next section. The major features of the model are the following:

(a) The β-pleated sheets are placed face to face with the strand directions, making a right angle. *Two of the strands, labeled a and d, form part of both β sheets* and join them covalently at two diagonally opposite corners. Each β sheet has the normal twist. [This twist is right-handed when the β sheet is viewed from an end, as for the top β sheet of Figure

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