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Synthesis of "Long," Hydrophilic, Protein-Cross-Linking Reagents

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Since most of the protein cross-linking reagents in use are strongly hydrophobic, their length cannot be increased beyond approximately 20 Å between the protein-reactive groups, before denaturation of most proteins becomes noticeable at already a very few cross-links per molecule. The synthesis of longer reagents, coupling to lysine or cysteine side chains, and containing strongly hydrophilic oligoproline chains, is described. As they bear an azodye, linking the oligoproline parts, the cross-links effected are amenable to a mild cleavage by reduction with dithionite. A trifunctional reagent was constructed by reacting trimesinic acid chloride with β-alanine ethyl ester; the carboxyl groups of this amino acid could then be activated for protein cross-linking by reactions leading to the hydrazide, and azides.

To compare the new reagents with the compounds in use at present, they were tested out on hemoglobin. The amount of reagent molecules coupled to the protein, and the fractions bifunctionally attached, as well as interchain linking were determined. The "long" reagents reached a distinctly higher efficiency in interchain cross-linking in this system, while showing smaller denaturing effects upon the protein. Thus, more than 11 reagent residues could be coupled to the hemoglobin tetramers without changes in its spectrum indicating denaturation of the heme environment, while shorter and more hydrophobic reagents had permitted the attachment of not more than four to six crosslinks.

A number of cross-linking reagents designed for applications in protein and enzyme chemistry have been proposed (1–6). All of them are bifunctional and make use of small molecules; the distance between the points of attachment on the surface of the protein will not exceed 18 Å in most cases (2). It has been found difficult to apply reagents of greater length to protein investigations. As their backbones usually consist of hydrophobic substances, they tend to denature proteins. Usually, these structures also permit folding or coiling, lowering the fraction of true-distance cross-links.

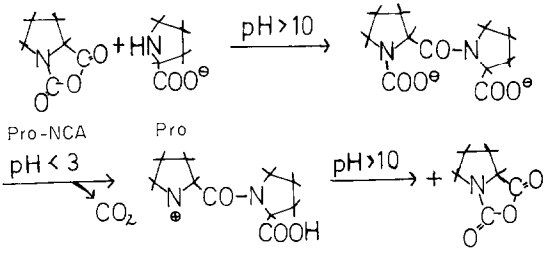
In previous communications (6,7), we described the cross-linking of several tertiary structures with the aid of these shorter bifunctional reagents. The investigations served the purpose of ascertaining distances between some characteristic side chains on the surface of these proteins. It was also shown, however, that even a rigid reagent of 15 Å length formed only a very small fraction (3% of the total) of cross-links between different subunits of hemoglobin, although the four subunits of this quaternary structure possess intimate contacts in a very compact globule.

The result indicated that the intention of cross-linking complex and more loosely grouped aggregates of protein molecules, as in biological membranes, must make use of much longer reagents. Moreover, they should be hydrophilic in nature. As a rough estimate, structures of at least 50 Å should be applied, and they should be fairly rigid.

We have developed a series of reagents with rigid noncoiling backbones on the basis of oligoproline helices, which may be varied between 30 and more than 100 Å in length. A trifunctional hydrophilic reagent is also described.

MATERIALS AND METHODS

Oligoprolines were synthesized by a modification of the NCA (α-*N*-carboxyanhydride) method of Hirschmann (8) and J. Engel (private communication). By stepwise reaction, proline residues were attached to the amino end of the growing oligopeptide chain:



In a typical experiment, 20 g of proline (Schuchardt Co., Munich) were dissolved in 500 ml of 0.45 M boric acid–NaOH–buffer, pH 10.2, and 25 g of proline-*N*-carboxyanhydride were added, while the solution was stirred with the aid of a turbine stirrer at 3000 rpm. The temperature did not exceed 4°C in this and all following steps. The pH was kept at 10.2 by the addition of 40% NaOH. When the reaction slowed down, the solution was acidified to pH 3.0, employing concentrated HCl solution. The evolution of carbon dioxide usually was completed after 20 min, when the pH was brought to 10.2 again. The same amount of proline-NCA

was then added. Coupling and decarboxylation were repeated in this manner until the volume became too large and temperature control too difficult for good yields. After this method, ten coupling steps could be completed with fair results. The solution was then neutralized to pH 7.0, and the mixture was brought to dryness in a rotatory evaporator. The crystalline residue was extracted twice with 2000 ml of dried ethanol, and the combined extracts were again brought to dryness. The peptide mixture was redissolved in 1.8 ml of H_2O/g, and 5 g each were fractionated on a P2 (BioRad) column, 3 × 210 cm, equilibrated with water. Fractions of the eluate (3 ml) were analyzed by paper electrophoresis in 0.1 M acetic and formic acid buffer, pH 1.9. After drying, the sheets were sprayed with *tert*-butylhypochlorite–toluidine (9). The oligomers between Pro_1 and Pro_{12} formed sharp bands. Usually, the electrophoresis patterns of this first chromatography showed the presence of 4–5 oligomers in each fraction (Fig. 1). Selected fractions (for instance, $Pro_{5,6,7,8}$ to $Pro_{6,7,8,9,10}$) were combined, brought to dryness, and again subjected to chromatography over the same column. Fractions of the second run (2 ml) were not only spotted for direct analyses, small samples were also subjected to dansylation (10), and run in the same buffer. In this case, two or three of the oligomers were usually present, and their relative amount was measured with the aid of a densitometer (Vitatron) in electrophoreses of dansylated samples. In some cases, the fluorescent bands were eluted from cut-out paper strips, and the fluorescence of the separated dansyl-oligoprolines was measured, after dilution to equal volumes, in a Zeiss-spectrofluorometer, as a control. Selected fractions were again combined, lyophilized, and the distribution of the oligomers was measured after dansylation of a sample. After this procedure, a fraction containing $Pro_{6,7,8}$ at a ratio of 14:66:20, was obtained in a 28% yield from the amount of proline and pro-NCA invested, and was used for subsequent syntheses. Similar amounts of $Pro_{4,5,6}$, $Pro_{8,9}$, and Pro_{9-13} were isolated.

From the isolated oligomers, larger oligoprolines could be synthesized either by using the fractions as starting material for stepwise synthesis as described above, or by combining two oligoprolines after the azide coupling method described in Results. In the latter case, due to the relatively good coupling yield, the separation of excess starting material from the product of doubled molecular weight was easily quantitative. Thus, in a typical experiment, it was possible to obtain Pro_{16-20}, with more than 70% Pro_{17-19}, in 81% yield from two pieces of $Pro_{8,9,10}$.

Ethylenediamine-1,2 was obtained from Merck Co., and redistilled at 10 mm pressure over a short fractionating column. Triethylamine was purified by distillation after refluxing over phthalic acid anhydride (20 g/liter) for 3-hr. Trimesinic acid, iodoacetyl chloride, and β-alanine were

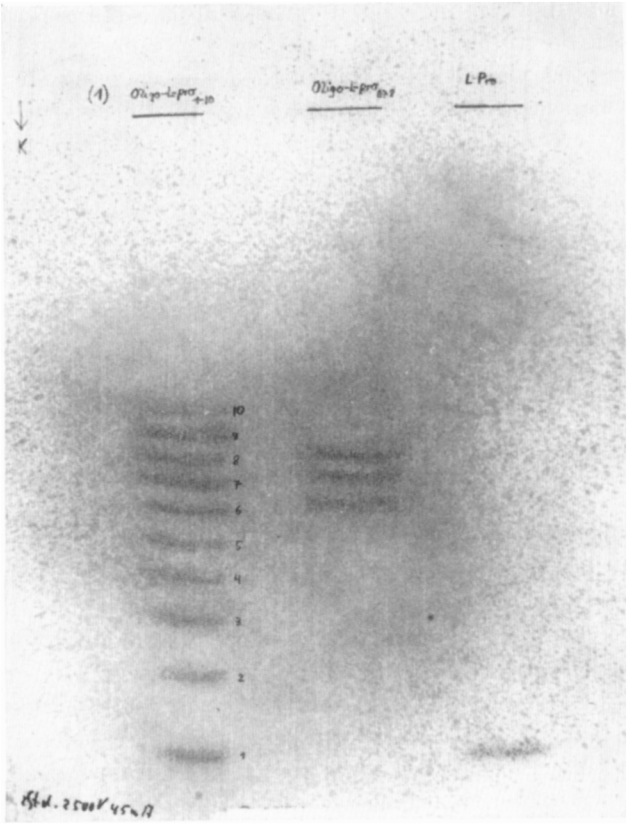


FIG. 1. Samples of the mixture of oligoprolines after nine coupling steps (left), of a fraction containing $Pro_{6,7,8}$ (middle) and of proline (right) were applied to the starting lines shown at the top. Electrophoresis ran for 2.5 hr at 30 V/cm in 0.1 M acetic acid–formic acid buffer, pH 1.9. The sheets were sprayed with *tert*-butylhypochlorite–toluidine (8).

products of Schuchardt Co. *p*-Diisocyanatoazobenzene was synthesized as described earlier (11), hemoglobin was prepared and stored as crystals suspended in saturated ammonium sulfate solution after the procedure of Rossi-Fanelli *et al.* (12). Circular dichroism (CD) spectra were taken with the aid of a Cary 61, at a concentration of 0.1 mg/ml and 1 mm path length. The amount of azo dye, incorporated into hemoglobin by the bifunctional reagents described, was measured spectrophotometrically after removal of the heme group, as described by Parek and Formanek (13). For quantitative determinations of amino acids by automated analysis in a Labotron micro amino acid analyzer, proteins were hydrolyzed for 22.5 hours in 6 N HCl in vacuum-sealed glass tubes.

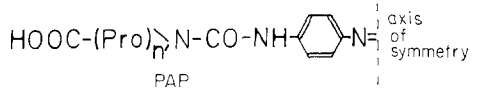
Sodium dithionite cleavage of azo dye compounds was effected as described previously (11). The resulting aromatic amines could be detected on paper electrophoreses by placing the sheets into a chromatography jar filled with nitrous fumes for a few minutes. The paper was then aired in a hood for at least 4 min, before spraying it with a 1% solution of *N*-napthylethylenediamine-1,2 in 0.1 N hydrochloric acid.

RESULTS AND DISCUSSION

Oligoproline pieces were chosen as part of the backbone of the new long hydrophilic bifunctional reagents, as these peptides form rigid helices, and their structure is well defined. With increasing molecular weight, the possibility of their forming random structures very rapidly decreases, and already tetra- and pentaproline show the typical CD spectra of the polyproline helices (14). Higher oligomers occur in only two forms: In water or media of similar strongly polar structure, the stretched-out helix II is stabilized by solvatation of the accessible peptide bonds, each proline residue contributes 3 Å to the length of the rod; in apolar solvents, the shorter helix I with less accessible peptide bonds is formed (15). Interconversion between these two forms, induced by changes of the solvent, occurs in a cooperative manner, model-building confirms the experimental finding that no third structure is possible (16).

In designing the bifunctional reagents, an azophenyl group was introduced between two pieces of oligoproline II helices, as the azo bond may be cleaved by dithionite under mild conditions without concomitant lysis of peptide bonds or even denaturation of proteins. As it turned out, the strongly hydrophilic nature of the oligoproline helices sustained the easy solubility of the reagents in water in spite of this hydrophobic centerpiece.

A facile synthetic approach to this basic structure lay in the reaction of *p*-diisocyanatoazobenzene with the oligoprolines, to form *p*-bis-(ureido)oligoprolylazobenzene (PAP):

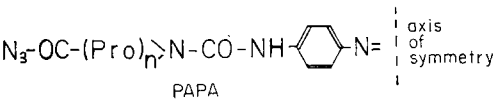


PAP

In a typical experiment, 6.1 g of oligoproline$_{6,7,8}$ was dissolved in 350 ml of dry chloroform, and 60 ml of freshly distilled dimethylformamide was added. After cooling the solution to 0°C, a solution of 530 mg of *p*-diisocyanatoazobenzene in 50 ml of dry chloroform was stirred in during 20 min. The reaction was completed at room temperature during 1 hr. The solid residue after rotatory evaporation of the solvent was suspended in 25 ml of glacial acetic acid, and stirred for 2 hr at room temperature. The

clear solution was then brought to dryness again, the oily residue was dissolved in 50 ml of water, and the pH was brought to 7.0 with 1 N NaOH. Small impurities of lower molecular weight azo dye derivatives and the excess of oligoproline were removed by chromatography over a Biogel P 2 column (50 × 3 cm) in water. Purity of the fractions was controlled by electrophoreses in 0.1 M pyridine–acetate buffer, pH 6.5. The product was lyophilized and formed yellow, pseudocrystalline needles. The CD spectrum indicated the presence of form II oligoproline. After splitting the azo bond by sodium dithionite as previously described (11), a single band was detected in electrophoresis at pH 1.9, identified as an aromatic monoamine as described in Methods. Moreover, a sample of the PAP band was eluted from paper electrophoresis, hydrolyzed, and the proline content was determined in automated amino acid analysis. Its value corresponded well with the expected amount calculated from the azodye extinction value (14.2 μmoles of proline/μmole of azodye). The yield of this reaction was usually more than 90%.

Conversion to a bifunctional acyl azide reagent for lysine side chains (PAPA). As the very rigid structure of oligoproline distinctly restricts the reactiveness of the terminal carboxyl groups, it was found difficult to obtain derivatives of PAP that would allow an easy acylation of lysine side chains in aqueous media. Thus, quantitative conversion to the bis-*p*-nitrophenyl ester, or direct coupling with the aid of water-soluble carbodiimides could not be achieved. Finally, reaction of the carboxyls with proteins via their azides reached good yields:



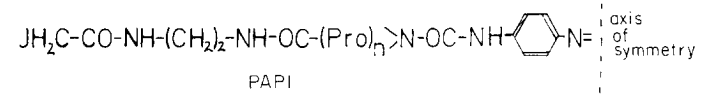
PAPA

To this end, 300 mg of PAP were mixed with 360 mg of dry triethylamine, and 18 ml of chloroacetonitrile were added. After a few minutes, the temperature rose to approximately 40°C, and a clear solution was obtained. The mixture was kept at 40°C for an hour, and brought to dryness in a rotatory evaporator. The dry residue of the bis-cyanomethylester was dissolved in 2 ml of ethanol, and diluted with 3 ml of water. After cooling to 0°C, 0.5 ml of 80% hydrazine hydrate were added. The reaction was allowed to proceed in the cold for 1.5 hours. After acidifying the mixture to pH 3 with 2 N HCl solution, the bis-hydrazide of PAP was separated out by chromatography over a 80 × 3 cm column of Biogel P 2, equilibrated with 0.01 M acetic acid, and lyophilized.

Just prior to protein coupling experiments, 10 μmoles of PAP-bis-hydrazide were dissolved in 0.3 ml of 0.5 N HCl solution in the cold, and 1.4 ml (20 μmoles) of sodium nitrite, dissolved in 0.1 ml of water, were

added. After 5 min at 0°C, a small grain of urea was dissolved, and the pH was brought to 3.5 cautiously with triethylamine.

Synthesis of PAP-bis-1-iodoacetamido-2-ethylamine (PAPI). To obtain a bifunctional reagent for cysteine side chains, both carboxyl groups of PAP were linked to ethylenediamine-1,2 in a first step, thus placing two amino groups at the end of the molecule. To this purpose, 300 mg of PAP were esterified with a mixture of triethylamine and chloroacetonitrile, as described above. The dried bis-cyanomethylester was taken up in a small volume of dry ethanol, and diluted with 5 ml of ethylenediamine. After 2 hr at room temperature, the solution was brought to a small volume in a rotatory evaporator, neutralized with hydrochloric acid and chromatographed over a 3 × 120 cm column of Biogel P 2, equilibrated with 0.01 M acetic acid. The first band eluted from the column was collected and lyophilized. The resulting diamine was then acylated with the aid of freshly distilled iodoacetyl chloride. The procedure followed the method described earlier (17), but sodium bicarbonate was used to neutralize the hydrochloric acid set free during the reaction.



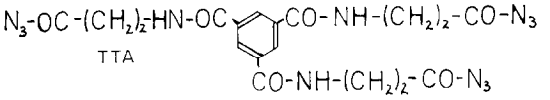
PAPI

The bis-iodoacetamide was again purified by chromatography over Biogel P 2. Paper electrophoreses at pH 1.9 and 6.5 were used to detect any incompletely acylated material, the purity of the compound was further established by cleaving the azo bond with the aid of sodium dithionite, and revealing a single aromatic amine in electrophoresis at pH 1.9, as described in Methods.

Synthesis of a trifunctional reagent. Most bifunctional reagents are fairly flexible, and afford crosslinking at a variety of smaller distances within their full span. Only occasionally a bridge is formed between different tertiary structures of a protein, for example between two subunits within a quaternary structure or between two adjacent proteins in a membrane. As shown for the example of a rigid 12–18 Å reagent, more than 90% of the cross-links lie within the same tertiary structure in the case of hemoglobin (7). A typical reagent of this kind is suberimidate (18), usually applied in large excess of the protein to be crosslinked. Advantages of these small reagents lie in their quick and easy occasionally radioactive synthesis, and the fact that the protein reaction products, though usually denatured, are not insoluble in aqueous media.

For this kind of nearest neighbour crosslinking a trifunctional reagent would distinctly ameliorate the chances of intermolecular links even at

the same size and concentration of reagent. We sought to maintain a facile synthesis. The maximum span between the reactive groups was to be 18 Å or more, and the reagent should have a hydrophilic structure, at least in parts. Therefore, trimesyl-tris-β-alanylazide (TTA) was chosen. The reagent might easily be lengthened by substitution of another hydrophilic oligopeptide for the β-alanine moieties.



TTA

Ten grams of trimesic acid were slowly dissolved in thionyl chloride (100 ml) by boiling under reflux. A slight turbidity was filtered off, and the solution was brought to dryness in a rotatory evaporator. The resulting oil solidified into white crystals at −10°C. β-Alanine ethyl ester (3.66 g), set free from its hydrochloride after the method of Hillmann (19) and 3.2 g of triethylamine were dissolved in 100 ml of dry dioxane. Trimesic acid trichloride (2.64 g), dissolved in 25 ml of dioxane, was slowly added to the solution with stirring. The addition took about 1 hr. The turbid solution was filtered, and evaporated to dryness. The resulting syrup was dissolved in 10 ml of ethanol, and 2.5 ml of hydrazine hydrate was added. The mixture was heated under reflux in a boiling water bath for 2 hr and cooled in an ice bath. The white crystalline mass was filtered off, and the trihydrazide was recrystallized from concentrated ammonia and water–dioxane, to a constant melting point of 163–165°C.

Analysis: Calculated for $C_{18}H_{27}O_6N_9 \cdot H_2O$. Found: C = 44.72%; H = 6.00%; N = 26.09%. C = 44.52%; H = 6.12%; N = 26.35%.

Just prior to protein cross-linking, 40 mg of the hydrazide was dissolved in 0.5 ml cold 1 N HCl and 40 mg of sodium nitrite in 0.4 ml of water were added with shaking in an ice bath. After a few minutes, a small crystal of urea was added. In protein cross-linking experiments where low ionic strength was a necessary condition, the azide was extracted from the solution with three 1-ml portions of ethyl acetate. The combined organic layers were quickly dried over magnesium perchlorate, rotated down to approximately 0.5 ml, and added to the protein solution with stirring. Usually, the aqueous azide solutions were cautiously brought to pH 3 with a few drops of triethylamine in the cold and added to the buffered protein solution.

Test reactions. In preparation of protein cross-linking experiments, the reactions involved were tested out with low molecular weight model compounds.

Thus, the bifunctional iodoacetamide derivative of PAP (PAPI) was

coupled to glutathione in the reduced form. Five micromoles of the sulfhydryl peptide was dissolved in 0.5 ml of water, the pH was adjusted with 0.1 ml of 0.5 M potassium phosphate, pH 7.4. One micromole of PAPI dissolved in 0.15 ml of water was added. After 20 min, samples of the mixture were applied to paper electrophoresis at pH 6.5. The band of unreacted glutathione ran well in front of the reaction product, while the control of the bifunctional reagent moved only a short distance with endosmosis. The reaction product, as well as a control strip of paper towards the glutathione band, were both cut out, and eluted with water. While the hydrolysis of the control band eluate contained only traces of various amino acids, the product band hydrolysis yielded the components of glutathione and proline at a ration indicating a bifunctional reaction: Carboxymethylcysteine (3.2 nmoles), glutamic acid (3.7 nmoles), proline (25.2 nmoles), and glycine (3.4 nmoles).

To test the efficiency of the reaction of the PAP azide and TTA reagents with amino groups, ethylenediamine-1,2 was chosen as the low molecular weight model compound. The TTA solutions, each prepared from 8 mg of hydrazide, were added to solutions of 30 mg of ethylene diamine in 0.05 M sodium phosphate-HCl-buffer at pH 8.0, varying the total volume between 2.8 and 300 ml, in the cold. After 20 min, samples of the reaction mixtures were applied to paper electrophoreses at pH 1.9. Bands corresponding to mono-, bi-, or trifunctional reaction with the diamine were easily separated by this method and were detected with ninhydrin. Densitometry gave at least a rough estimate of the relative amount of the three products. The trifunctional coupling product was identified by comparison with the result of a reaction of the azide with the diamine in dry ethyl acetate. Trimesic acyltri-β-alanine was run as a control, and detected by the *t*-butylhypochlorite–toluidine spray cited in Methods; it was not found on the electrophoreses of this series, however.

As a result, a practically quantitative reaction of the three activated carboxyl groups with protein side chains under these conditions could be predicted. As an estimate, the concentration of amino groups in a usual protein cross-linking experiment was assumed to amount to 10^{-2}–10^{-4} M. At the lowest concentrations of ethylene diamine amino groups employed, 10^{-5} M, only 20% of bifunctional, and 5% of monofunctional reaction product was found, as compared to 75% of the trifunctional derivative. At an amino group concentration of 10^{-4} M, only small amounts of the bifunctional and monofunctional coupling products were found.

Similar experiments were performed with the same result on the oligoproline reagent described above. Thus, the azide activated reagents for protein amino group cross-linking are suitable for reactions under plausible protein concentrations, with good yields.

Cross-linking of a model protein: hemoglobin. The reagents described here had been designed especially for the cross-linking of different protein molecules lying in neighbouring position in natural structures, as membranes or contractile protein systems. Hemoglobin was chosen as a test protein for this intermolecular cross-linking, as its four subunits present a good example of four tertiary structures in close contact. The molecular dimensions are precisely known. In solution under physiological conditions, practically no dissociation is observed. After forced dissociation into monomers, it is fairly easy to determine the relative amounts of intra- and interchain cross-links, as the artificial dimers, usually containing one α- and one β-chain, can be separated from monomers. To measure the efficiency of a cross-linking reagent, it is then necessary to determine the amount of reagent molecules bound to monomers and dimers, and the amount only monofunctionally attached.

This kind of experiment, moreover, had been performed on hemoglobin in previous experiments, making use of an azo dye reagent with a maximal span of 18 Å (7). Most of the cross-links were intrachain, attached to two amino acid side chains of one β subunit. Less than 3% of the cross-links formed bridges between two different tertiary structures. In comparing the relative rates of intrachain and interchain cross-linking, therefore, the hemoglobin examples offered a good possibility of assessing the influence of widening span of the reagent.

Human hemoglobin was prepared as cited in Methods and dialyzed thoroughly against water. The stock solution contained 1 μmole of the protein in 1 ml. Four milliliters was diluted with an equal volume of 0.2 M sodium phosphate–HCl buffer, pH 8.0, and 12–40 μmoles of freshly prepared PAPA or trimesyltri-β-alanylazide was added with stirring at 20°C. The pH was kept constant for 20 minutes, then the mixture was passed quickly over a Sephadex G 50 column (30 × 50 cm), equilibrated with 0.01 M sodium phosphate–HCl buffer at pH 7.4. The absorbancy spectrum as well as the oxygen binding curve (7) of the modified hemoglobin was not changed by this procedure.

To evaluate the amount of reagent covalently linked to the protein, completely dialyzed samples were subjected to hydrolysis and amino acid analyses, after their protein content had been determined spectrophotometrically (20), and after the method of Lowry *et al.* (21). In the case of PAPA modifications, the addition in proline content permitted the calculation of the number of the attached reagent molecules per molecule of hemoglobin. The amount of β-alanine, on the other hand, appearing a short distance after phenylalanine in the usual amino acid diagrams, gave corresponding values for modification with the trimesic acid reagent. In both cases more than 84% of the reagent was covalently

bound to the protein at a threefold molar excess. At tenfold molar excess, 65% of both substances were coupled to the protein. Thus, at threefold molar excess of the reagents, each protein molecule carried 2.7 residues of the modifying agent, while at tenfold molar excess, 6.5 residues of the reagents were incorporated into each hemoglobin tetramer.

In the previous experiments (7) on hemoglobin cross-linking, using short bifunctional azo dye reagents, the heme spectrum had indicated a beginning denaturation of the protein when more than 6 cross-links per tetramer were introduced. In sperm whale myoglobin, more than four reagent residues per molecule gave rise to denaturation (4,6). Therefore, in consecutive experiments, the concentration of the TTA reagent was raised until 15 reagent residues had been introduced per hemoglobin tetramer. At the point of attachment of 13 reagent molecules, the heme spectrum showed the characteristic lowering of the peak at 576 nm with concomitant broadening of the 540 nm peak, indicating a denaturation of the heme environment. Thus, the hydrophilic reagents permit a much higher degree of cross-linking, before denaturation becomes distinct.

On the other hand, lower stages of cross-linking in this manner seemed to have a stabilizing effect upon hemoglobin. For these measurements, hemoglobin carrying 4 TTA cross-links, of which 2.1 were interchain links, was brought to a concentration of 2 and 4 mg/ml in several series of experiments, and dimethylformamide was added to aliquots to final concentrations between 3 and 30%, in 10 steps. The mixtures were incubated for precisely 10 min at 41°C, and the absorbancy spectrum between 590 and 500 nm was quickly recorded. Control experiments used the same hemoglobin preparation without cross-linking. While the first spectral changes—a lowering of the extinction at 576 nm and broadening of the peak at 540 nm, as well as a shift of this peak towards longer wavelengths—were recorded at 20% dimethylformamide concentration, the spectrum of the cross-linked protein remained unchanged up to 26.5% dimethylformamide content.

The application of PAPA permitted a direct determination of the relative amount of bifunctionally and monofunctionally coupled reagent molecules. After removal of the excess reagent by gel filtration, the modified hemoglobins were incubated with sodium dithionite to cleave the reagent azo groups, as previously described (11). The pH of the mixtures was then adjusted to 4.5, and through dialysis all free halves of monofunctionally attached reagent molecules were removed. Amino acid analyses were then performed in comparison with material isolated before dithionite cleavage, and the difference in proline content was made use of to calculate the monofunctionally attached fraction. Between 45 and 60% of the reagent molecules were true cross-links after

these determinations. Shorter reagents of similar reactivity (7) had reached a higher percentage of bifunctional cross-linking (60–65%). However, the probability of a fairly rigid and long cross-linking reagent of 55–65 Å average length in reaching suitable partners after its first, one-armed attachment to the protein molecule, roughly 60 Å in diameter, and with a curved surface, is probably lower than for a reagent of 12–18 Å length. On the other hand, the percentage of interchain cross-links for the longer reagent should be distinctly higher.

Therefore, the amount of interchain cross-links was determined for both PAP- and TTA-modified hemoglobins. After removal of excess reagent as described above, the combined fractions containing hemoglobin were adjusted to pH 8.0. *p*-Chloromercuribenzoate (50 mg), dissolved at pH 9.0 in 3 ml of water with the aid of sodium hydroxide, was added, and finally sodium dodecylsulfate was stirred in to a final concentration of 0.01%. The mixture was left to stand overnight in the cold room, and passed over a Sephadex G-75 column (2.5 × 50 cm), equilibrated with 0.01 M sodium phosphate–HCl buffer at pH 7.4, containing 0.01% sodium dodecylsulfate. Under these conditions, sperm whale myoglobin and horse hemoglobin both gave a single sharp peak with identical elution volume. The cross-linked hemoglobins, however, were eluted in two peaks, the first corresponding to a molecular weight of 35,000, the second at the elution volume of myoglobin. The areas of both peaks were determined from the LKB Uvicord diagrams, and from the relative amount of the artificial dimers and of monomers, the number of interchain cross-links per molecule of hemoglobin was calculated. The resulting figure corresponds to the lower limit of interchain cross-links, as a fraction of the artificial dimers will contain more than one reagent residue attached to both peptide chains.

Table 1 collects the data on total residues introduced, bifunctional PAPA cross-links, and percentage of artificial dimers obtained. From these results a minimum rate of 15% of true interchain linkages from the total of reagent molecules coupled to hemoglobin in the case of PAPA reagent, and of 24% in the case of trimesyltris-β-alanylazide was calculated.

Compared with the results of shorter reagents (7), coupled to hemoglobin by utilizing isocyanate, isothiocyanate, or iodoacetamido groups, where the rate of interchain cross-linking, measured in the same manner as described here, was 3% of true bifunctional cross-links, which in turn corresponded to 60–70% of attached reagent molecules, the greater length of the new reagents achieved a distinctly higher degree of "long" cross-links, even though hemoglobin has the disadvantage of a relatively high content of lysine side chains.

TABLE 1
Reaction of Bifunctional Reagents with Hemoglobin[a]

Reagent	Final concentration mM	Reagent residues introduced/ molecule of protein	Bifunctional coupling	Artificial dimers, % of total residues introduced
PAPA	1.2	2.6	1.6	36
	2.0	4.1	2.0	30
	2.5	4.8	2.2	25
	3.0	5.4	2.5	20
	4.0	6.5	2.9	15
TTA	1.2	2.6		69
	2.0	4.2		39
	2.5	4.8		32
	3.0	5.6		28
	4.0	6.5		24

[a] The protein was dissolved in 0.08 M phosphate-HCl buffer, pH 8.0, at 0.4 mM final concentration.

The yield of linkages between different tertiary structures can be very much higher when the azides are brought to react with less basic proteins, or when reagents for less frequent side chains, as SH groups are employed. This was demonstrated by the cross-linking of ferritin subunits by PAPA, or F-actin from rabbit muscle, in which all subunits carry one SH group on their surface (17) by PAPI. Trimesyltris-β-alanylazide, on the other hand, was used to link the amino ends of some three-stranded collagen model oligopeptides (J. Engel, private communication).

ACKNOWLEDGMENTS

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