A DISULFIDE-BRIDGE BIFUNCTIONAL IMIDOESTER AS A REVERSIBLE CROSS-LINKING REAGENT*

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SUMMARY. A disulfide-bridged bifunctional imidoester, dimethyl 3, 3' dithiobispropionimidate (DTP) has been prepared and investigated as a reagent to introduce covalent cross-links in proteins that can subsequently be broken by mild reduction. Such reversible cross-links were shown to be introduced by DTP in the soluble subunit proteins aldolase and Concanavalin A. DTP was also used to modify human intact erythrocytes. Such modification rendered the erythrocytes resistant to hypotonic lysis; subsequent treatment with mercaptoethanol lysed the cells. After DTP-modification of the cells, the hemoglobin contained in them could still be reversibly oxygenated and deoxygenated.

Bifunctional imidoesters (1-3) have been successfully used as cross-linking agents in protein chemistry. The mild conditions under which imidoesters react, and their specific conversion of free ammonium groups to amidinium residues with retention of ionic charge, produce little or no detectable structural changes in proteins (4, 5). This makes bifunctional imidoesters very useful. In order to demonstrate, however, that a particular biochemical consequence of a reaction with a bifunctional imidoester is due to cross-linking, and not to the chemical modification itself, it would be useful to employ a reagent which produced cross-linkages that could subsequently be broken by mild treatment. To this end, we have synthesized and studied some of the reactions of dimethyl 3, 3'-dithiobispropionimidate

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(DTP) (Fig. 1 top), and their reversal by sulfhydryl compounds. Our specific purpose in making DTP was to use it to study the effects of cross-linking and its reversal upon the catecholamine-stimulated adenylate cyclase activity in pigeon erythrocyte membranes. This aspect of our studies will be reported elsewhere (A. Ruoho and S. J. Singer, to be published). During the course of this work, we became aware of similar studies by Wang and Richards (6).

MATERIALS AND METHODS

Synthesis of Dimethyl 3, 3'-dithiobispropionimidate Dihydrochloride. Dimethyl 3, 3'-dithiodipropionimidate dihydrochloride was prepared via the dinitrile by a modification of the method employed by Johnson and Gallagher (7) for the synthesis of the diethyl analog. To a rapidly stirred mixture of 30 g (0.75 mole) of NaOH, 150 ml of water, and 150 ml of CH_2Cl_2 at 0° was added 51 g (0.31 mole) of S-(2-cyanoethyl) isothiouronium chloride (8) all at once. Iodine (39 g, 0.153 mole) was added in portions over a 20 min. period until the brown color dissipated only slowly. After a further 10 min. of maintaining the brown color, the phases were separated and the organic layer washed twice with 2 *N* NaOH and once with brine, dried over K_2CO_3, and concentrated under reduced pressure to afford 21.6 g of light pink crystals. Recrystallization from ether afforded 19.3 g (73% yield of 3,3'-dithiodipropionitrile as white prisms, mp 48-50° [lit. (8) 49-51°], in two crops. A solution of 1.03 g (6.0 mmoles) of the dinitrile, 0.53 ml (13 mmoles) of dry methanol, and 5 ml of dry $CHCl_3$ was protected from moisture and stirred at 0° while dry HCl gas was bubbled in. After 0.5 g of HCl had been absorbed, the mixture was stored in the cold for four days. The white solid was isolated by filtration under a blanket of nitrogen, washed with dry ether-chloroform (1:1) and dry ether, and dried over P_2O_5 in a vacuum desiccator to give 1.80 g (99% yield) of dimethyl 3, 3'-dithiodipropionimidate dihydrochloride as a white powder, m.p. 174-6° (softens 170°) [lit. (9) 125-8°, 175-6°]. Analysis: calculated for $C_8H_{18}Cl_2N_2O_2S_2$: C: 31.06%; H:5.87%; Cl:22.93%; S:20.74%; found: C:30.85%; H:6.06%; Cl:23.10%; S:20.98%. A portion

of the dihydrochloride was converted to the free base by dissolving in chloroform with triethylamine, washing twice with 0.1 *N* NaOH, then drying and concentrating under reduced pressure. The nmr spectrum of this colorless oil showed no resonances other than those at δ2.77 ppm (m, 4H, $-CH_2CH_2-$), δ3.70 ppm (s, 3H, CH_3O-), and δ6.7 ppm (broad, 1H, = NH); and the infrared spectrum showed a strong absorption at 1658 cm^{-1} (C = N) with no evidence for nitrile (at 2250 cm^{-1}) or amide II bands (at 1570 cm^{-1}).

Dimethylsuberimidate (Fig. 1, bottom) (DMS) was synthesized by the

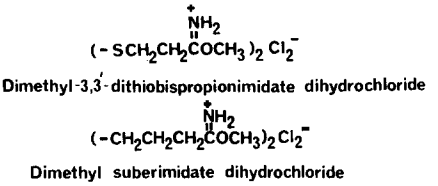

Fig. 1 - Formulae of two bifunctional imidates.

method of Davies and Stark (3).

Cross-linking of Soluble Proteins. Rabbit skeletal muscle aldolase (Sigma) at a concentration of 1.5 mg/ml in 0.05 M Tris-HCl, 1.0 M NaCl buffer, pH 7.4, and concanavalin A (Miles-Yeda) at 2.0 mg/ml in 0.05 M Tris-HCl buffer, pH 7.4, were incubated for 3 hr and 1 hr, respectively, at 30°C with either 3 mM DMS or 3mM DTP. A portion of each aldolase reaction mixture was then brought to 0.1 M in dithiolthreitol (DTT) for 1 hr at 37°C; a portion of each Con A reaction mixture to 0.4 M DTT for 3 hrs at 37°C. All aldolase mixtures were electrophoresed in 5.25% polyacrylamide gels and Con A mixtures in 3.25% gels containing 0.1% sodium dodecyl sulfate (10). Aldolase samples were prepared for electrophoresis by a 5-fold dilution into 5% SDS and were incubated for 10 min at 37°C. Con A samples were treated similarly, but were then dialyzed against 10 mM Tris HCl buffer, pH 7.4, containing 0.1% SDS to remove excess salt. To the reduced Con A samples 0.1% β-mercaptoethanol was added to prevent reoxidation.

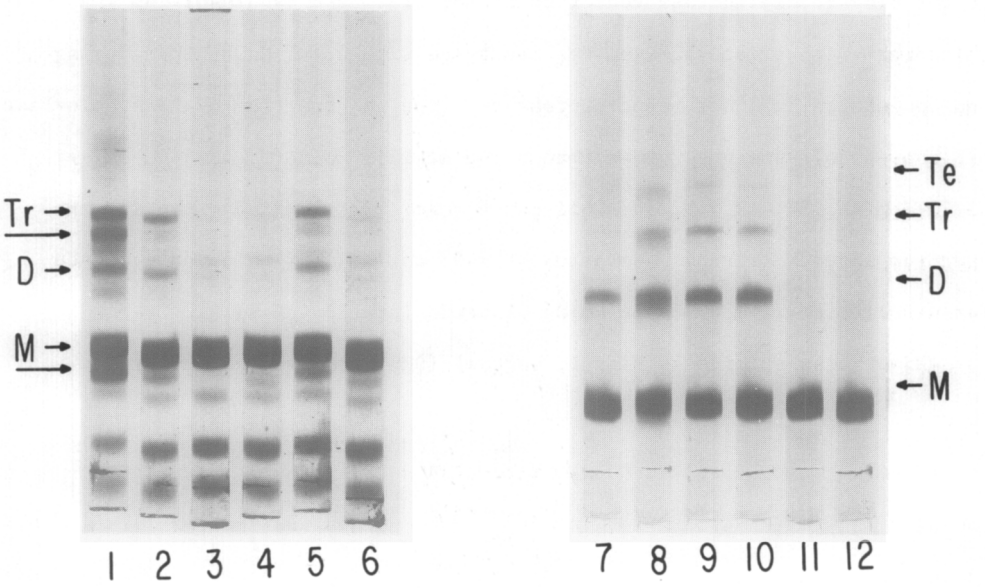

Fig. 2 - SDS-polyacrylamide gel electrophoresis patterns of Concanavalin A samples (1-6) treated as follows: 1) DTP alone; 2) DMS alone; 3) unreacted control; 4) unreacted control + DTT; 5) DMS-treated, followed by DTT; and 6) DTP-treated, followed by DTT. Aldolase samples (7-12) treated as follows: 7) DTP-treated, followed by DTT; 8) DTP alone; 9) DMS-treated, followed by DTT; 10) DMS alone; 11) unreacted control + DTT; 12) unreacted control.

Reaction of Human Erythrocytes with DTP. Washed fresh human erythrocytes were carefully brought to 0.02M in DTP in 310 imosm phosphate buffer, pH 7.4, and allowed to react for 20 hrs at 5°C. After thorough washing, the cells were examined for their lytic properties by suspending them in water for 5 min, then centrifuging, and measuring the absorption of the supernatant at 540 nm. To examine the oxygen binding properties of the DTP-modified cells, the absorption spectrum of a well-aerated sample of the cells was measured against air in a Unicam double-beam recording spectrophotometer (SP-800). To this sample was then added 5 mg of solid sodium dithionate and immediately the spectrum of the deoxygenated hemoglobin was recorded. The sample was then reoxygenated by shaking with air, and the spectrum recorded again.

RESULTS AND DISCUSSION

The results of cross-linking experiments with the two soluble proteins Con A and aldolase are shown in Fig. 2. The gel patterns 1 and 2 demonstrate that after cross-linking with DTP or DMS, respectively, under the reaction conditions employed, Con A consisted primarily of monomer (M), dimer (D), and trimer (Tr) components. Treatment of these partially cross-linked Con A samples with DTT largely converted the DTP-modified sample back to monomer (compare 1 and 6) whereas the DMS-modified sample was unaffected (compare 2 and 5). These results were as expected. Unexpected, however, were the two extra peaks in DTP-treated Con A, migrating somewhat more rapidly than the monomer and trimer bands (long arrows in 1). These disappeared with the DTT treatment. This suggests that these molecular species might be due to some intrachain cross-linking reactions that prevent the Con A polypeptide from unfolding completely in the sodium dodecyl sulfate solution (11, 12).

Similar cross-linking with DTP and reversal with DTT was observed with aldolase (compare 8 and 7, Fig. 2). However, about 30% of the dimer band was not converted back to the monomer form upon reduction. The reason for this is not clear, but it raises the possibility that reversibility of the DTP cross-linking reaction may not always be complete, which may be an important consideration in its use.

Under identical conditions, DTP consistently produced a greater degree of cross-linking than did DMS. This is somewhat surprising since the reactivities of the imidoester groups of the two compounds should be identical, and may be due to restricted rotations around the S-S bond in DTP.

When DTP was used to modify human erythrocytes, the cells became resistant to hypotonic lysis, as we had previously found (1) with the bifunctional reagent diethylmalonimidate (DEM). After equivalent modification with the monofunctional reagent, ethylacetimidate, however, the cells could still be lysed in water. In the case of DEM, we have shown by means of the ^{14}C-labeled reagent (A. Dutton and S. J. Singer, to be published)

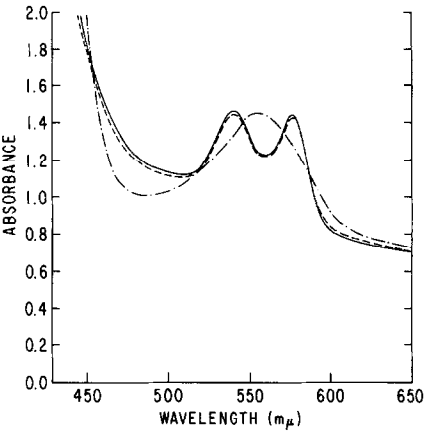

Fig. 3 - Absorption spectra of oxygenated (———), deoxygenated (—·—·—·) and reoxygenated (-----) hemoglobin in human erythrocytes reacted with DTP. See text for details.

that this lytic resistance is due to the cross-linking of the hemoglobin inside the cell, and this is most likely the case with DTP also. Treatment of the DTP-modified cells with 1% mercaptoethanol in the isotonic phosphate buffer for 10 min at 20°C caused the cells to lyse, indicating that the cross-linking by the DTP was substantially reversed. The aerated DTP-treated cells appeared slightly brown, but showed an essentially normal visible spectrum (Fig. 3) and could be deoxygenated and reoxygenated. These results show that although the hemoglobin was modified enough to cross-link it extensively, it could still undergo reversible oxygenation, attesting to the mildness of the DTP-modification reaction.

The potential usefulness, as well as some limitations, of DTP as a cross-linking agent in protein chemistry, and in particular in the determination of nearest neighbor relationships in complex protein aggregates, should be clear from these results.

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