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Obligatory Hybridization of Heterologous Immunoglobulin Light Chains into Covalently Linked Dimers†

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ABSTRACT: Immunoglobulin light chains, which generally show preference for homologous association in forming dimers, can be forced to hybridize with heterologous molecules in yields of 80–100%. Light chains (λ type) from the patient Mcg were S-sulfonated at the penultimate half-cystine residues of the interchain disulfide bond. The interchain half-cystine residues of λ- or κ-type light chains from a second source were reduced, and the two samples were mixed. A hybrid was produced with formation of an interchain disulfide bond between heterologous light chains. The Mcg light chain (Bence-Jones) dimer crystallizes readily, and several hybrids containing the Mcg light chain also crystallized. The affinity of the Mcg dimer for bis(dinitrophenyl)lysine was also passed on to the hybrids. For example, the Mcg × Weir hybrid showed binding affinity for the ligand, while the parent Weir dimer did not. The Mcg and Vil λ chains are identical in amino acid sequence in their first hypervariable regions and are similar throughout the variable domains. Both the Vil dimer and the Mcg × Vil hybrid bound bis(dinitrophenyl)lysine. The Tew κ chain, like the Mcg protein, was obtained from a patient with amyloidosis and included in its sequence all but one of the side chains associated with dinitrophenyl ligand binding in the Mcg dimer. The Tew dimer and the Mcg × Tew hybrid showed the expected binding of bis(dinitrophenyl)lysine.

The initial impetus for this work came from Dr. Elvin A. Kabat in 1977. He suggested that crystal-structure analyses of hybrids of the Mcg light chain and such closely related proteins as the Vil λ chain would contribute to the understanding of the specificity and geometry of complementarity-determining regions in immunoglobulins (Kabat et al., 1977; Fett & Deutsch, 1974, 1976; Ponstingl & Hilschmann, 1969).

The use of the Mcg protein for hybridization is advantageous because the three-dimensional structure has been determined and correlated with the amino acid sequence (Schiffer et al., 1973; Edmundson et al., 1975; Fett & Deutsch, 1974, 1976). Moreover, the binding of hapten-like molecules to the Mcg Bence-Jones dimer has been studied both in crystals and in solution (Edmundson et al., 1974; Firca et al., 1978).

The principal obstacle to success was the preference of light chains for self- (homologous) association rather than heterologous dimerization (Stevenson & Straus, 1968). In the absence of an intact interchain disulfide bond, light chains participate in monomer–dimer equilibria (Stevenson & Dorrington, 1970; Björk & Tanford, 1971). The dimer is stabilized by noncovalent interactions between pairs of like domains (i.e., V_l–V_2 and C_l–C_2 pairs[1]). Assuming that heterologous light chains form noncovalent dimers, even in minute amounts, we searched for a method to prevent dissociation by locking the components together with an interchain disulfide bond. In the present article we describe such a method and its application to both λ and κ chains.

Materials and Methods

Preparation of Bence-Jones Proteins. Patients with multiple myeloma and/or amyloidosis were screened for the presence of urinary Bence-Jones proteins. Urine was collected from patients in which the quantities of Bence-Jones protein exceeded about 1 g/L. The protein was precipitated by the addition of ammonium sulfate to 90% saturation. After centrifugation, the pellet was washed twice with 75% saturated ammonium sulfate and stored as an ammonium sulfate paste at −10 °C.

Samples of proteins or urines were kindly supplied by Drs. H. F. Deutsch of the University of Wisconsin (Mcg and Weir), N. Hilschmann of the Max-Planck Institute in Göttingen, West Germany (Vil), A. Solomon of the University of Tennessee (Hud and May), E. F. Osserman of Columbia University (Cot, Ste, Joy, and Tew), and T. M. Cosgriff (All) and B. D. Cheson of the University of Utah (Bla). The Tew protein was a κ chain and all other proteins were λ chains. Of the proteins for which partial or complete amino acid sequences are known, the Vil and Weir λ chains have been assigned to subclass II, the Mcg protein to subclass V, and the Tew κ chain to subclass II (Ponstingl & Hilschmann, 1969; Fett &

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[1] Abbreviations used: V, variable domain; C, constant domain; DEAE, diethylaminoethyl; MEA, 2-mercaptoethylamine; MAA, 2-mercaptoacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Nbs, 5-thio-2-nitrobenzoate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EDTA, ethylenedinitrilotetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Dnp, 2,4-dinitrophenyl.

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Deutsch, 1974, 1976; Putnam et al., 1973).

The purity of each protein was assessed by electrophoresis (see subsequent section). Samples found to be contaminated were purified by gel filtration or ion-exchange chromatography. Noncovalent and covalent Bence-Jones dimers were readily separated from aggregates, monomers, degradation products, and smaller contaminants at pH 8.0 and 4 °C on a 2.5 × 120 cm column of Sephadex G-100, with 0.05 M Tris-HCl and 0.15 M NaCl as eluant. For more complex mixtures the proteins were fractionated at 4 °C on 1.5 × 30 cm columns of DEAE-Sephadex A-50 or DEAE-Bio-Gel A, using linear salt gradients. In DEAE-Sephadex the gradient ranged from 0 to 0.3 M NaCl in 0.05 M Tris-HCl, pH 8.3; for DEAE-Bio-Gel A the concentration of NaCl was raised from 0 to 0.2 M in 0.01 M Tris-HCl, pH 8.3.

Attempts To Produce Noncovalent Hybrids. Initial attempts to produce hybrids involved procedures similar to those used in the interconversion of conformational isomers of Mcg light chains (Firca et al., 1978). Covalent dimers from two different sources were converted into mixed disulfides by disulfide interchange reactions (Smithies, 1965). One protein was derivatized with 2-mercaptoethylamine (MEA) to introduce positive charges, and the second was reacted with 2-mercaptoacetic acid (MAA) to add negative charges. Each noncovalent dimer was dissociated into monomers by dialysis against 0.4 M propionic acid for 4 h. The two samples were mixed, and reassociation was carried out by exhaustive dialysis at 4 °C against 4 mM sodium acetate, pH 5.4 (Stevenson & Dorrington, 1970). The reaction mixtures were examined by electrophoresis or chromatography on DEAE-Sephadex A-50.

Covalent Hybridization Reactions. (1) S-Sulfonation of the Interchain Disulfide of the Mcg Bence-Jones Protein. In a typical experiment, 12 mg of Mcg Bence-Jones dimer (concentration ~7 mg/mL) was reacted at 22 °C with 90 mM sodium sulfite and 1 mM Nbs_2 in 0.1 M sodium borate, pH 8.5. After 20 min the protein was separated from the reactants by gel filtration on a 0.9 × 25 cm column of Sephadex G-25, equilibrated with 20 mM Tes and 1 mM EDTA, pH 6.5. The eluant was previously deaerated and was kept saturated with nitrogen during the run. The protein was detected by automated absorbance measurements at 280 nm.

(2) Reduction of the Interchain Disulfide Bond of the Second Parental Species. Covalent dimers from patients other than Mcg were dissolved in concentrations of 10–25 mg/mL in 0.1 M sodium borate and 4 mM EDTA, pH 8.8. Each solution was made 0.1 M in 2-mercaptoethanol and maintained at 22 °C under a nitrogen blanket for 4 h in the dark. The reagents were removed by gel filtration on Sephadex G-25, and the protein was collected under nitrogen.

(3) Obligatory Covalent Hybridization. Equal quantities of the S-sulfonated Mcg protein and the reduced form of the second protein were mixed at 4 °C in pH 6.5 20 mM Tes–1 mM EDTA, which was 0.1 M in $SrCl_2$ (the sulfite "trap"). After the solution was mixed, its A_{280} value for the solution was 2.7. The mixture was kept at 4 °C under a nitrogen blanket in the dark for a period of time based on the kinetics of formation of each hybrid (usually 6 to 20 h). Reactants were removed by dialysis against Tes–EDTA buffer at 4 °C.

Kinetics of S-Sulfonation and Hybrid Formation. The kinetics of S-sulfonation were followed by measuring the incorporation of [^{35}S]sulfite (New England Nuclear) into the Mcg protein. At specified intervals the protein was precipitated with 10% (v/v) trichloroacetic acid (Cl_3AcOH) which was 90 mM in Na_2SO_3. The precipitates were washed onto Millipore filters (HAWP 02500) previously soaked in 10% Cl_3AcOH–90 mM Na_2SO_3 to reduce nonspecific binding of the radioisotope. The filters were placed in 10 mL of a solution of 7.0 g of 2,5-diphenyloxazole, 0.6 g of *p*-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 100 g of naphthalene in 300 mL of methyl-Cellosolve and 700 mL of toluene. Radioactivity was measured in a Packard Tri-Carb scintillation counter.

In our study of the kinetics of hybrid formation, we increased the concentration of $SrCl_2$ to 0.2 M. At various intervals the hybridization was terminated by the addition of 0.2 M *N*-ethylmaleimide (to derivatize the sulfhydryl group of the second parental species and inactivate the sulfite released during the reaction). The fraction of covalent hybrid dimers at each time point was estimated by $NaDodSO_4$ electrophoresis (see next section).

Electrophoresis of Parental Species, Hybrids, and Intermediates. Several electrophoretic systems were required to characterize the wide variety of proteins used in the present study. For routine assessment of the purity of parent Bence-Jones proteins, it was convenient to employ cellulose acetate membranes in pH 8.6 barbital buffer in a Beckman microzone apparatus. In homogeneity tests for parental species and hybrids under nondenaturing conditions, electrophoresis was performed in 1.5-mm slabs of 7.5% polyacrylamide gels with the pH 9.5 system of Davis (1964). The fractionation could be improved in some cases by including 8 M urea in a 4% resolving gel. The gels were stained in 0.04% (w/v) Coomassie brilliant blue G-250 in 3.5% (v/v) perchloric acid (Reisner et al., 1975; Holbrook & Leaver, 1976). Destaining was carried out in 5% (v/v) acetic acid.

Electrophoresis under more stringent denaturing conditions was performed in slabs of 15% gels with 0.9 M acetic acid and 6.3 M urea (Panyim & Chalkley, 1969). $NaDodSO_4$ electrophoresis was conducted in 10–12.5% gels in the presence or absence of 2-mercaptoethanol (Laemmli, 1970). The acid–urea and $NaDodSO_4$ gels were stained with 0.2% Coomassie brilliant blue in a solution of 45% methanol–7% acetic acid and were destained in 5% methanol–7% acetic acid. In time-course experiments the relative quantities of covalent hybrid dimers were estimated by scanning stained $NaDodSO_4$ gels with an Ortec densitometer.

Binding Studies with Bis(dinitrophenyl)lysine. The binding of bis(dinitrophenyl)lysine by the hybrids was compared with that of the native Mcg Bence-Jones dimer by the equilibrium dialysis method (Karush, 1962; Voss & Eisen, 1968; Firca et al., 1978). The spectrophotometric technique used to determine the quantities of bound ligand in our initial binding studies (Firca et al., 1978) was replaced by a procedure utilizing tritium-labeled bis(dinitrophenyl)lysine.

The labeled ligand was synthesized by reacting [^{3}H]lysine (New England Nuclear) with 1-fluoro-2,4-dinitrobenzene in 66% (v/v) ethanol buffered with sodium carbonate at pH 9 for 24 h at 22 °C in the dark. The derivative comigrated with bis(dinitrophenyl)lysine in two-dimensional thin-layer chromatography on polyamide sheets (Niederwieser, 1972). Concentrations of the derivative were determined spectrophotometrically at 342 nm in glacial acetic acid, using a molar extinction coefficient of 32 200 cm^{-1} (Fraenkel-Conrat et al., 1955).

Equilibrium dialysis was performed at 4 °C in microchambers with 50 μL of protein dimers (2.5 mg/mL) or bis(dinitrophenyl)[^{3}H]lysine per side. After 16–20 h of gentle agitation, two aliquots of 10 μL from each side were added to scintillation vials and diluted with 1 mL of water and 10 mL of 0.85% (w/v) Omnifluor (New England Nuclear) in a 1:2 (v/v) solution of Triton X-100 and toluene. The radioactivity

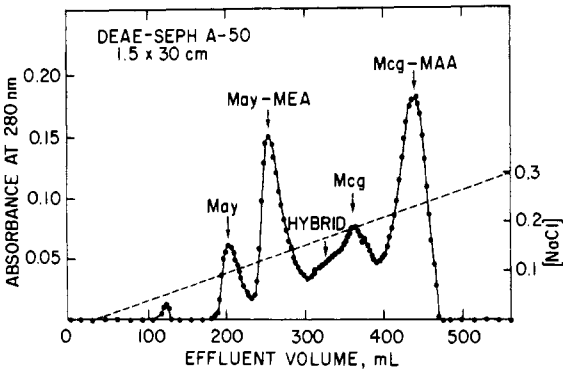

FIGURE 1: Chromatography of the reaction mixture after attempted noncovalent hybridization of the May and Mcg λ-type light chains. The 1.5 × 30 cm column of DEAE-Sephadex was equilibrated at 4 °C with 0.05 M Tris-HCl, pH 8.3. The proteins were eluted with a salt gradient (see dashed line on the effluent curve); fractions of 3 mL were collected. Components were identified by electrophoresis of the fractions marked with arrows. The covalent parental dimers are labeled May and Mcg. The positively charged mercaptoethylamine derivative is marked May-MEA and the negatively charged mercaptoacetic acid derivative is designated Mcg-MAA. The noncovalent hybrid (see shoulder on Mcg peak) was produced in yields of 10–15% by mixing the May-MEA and Mcg-MAA derivatives under dissociating conditions and then allowing the components to renature slowly.

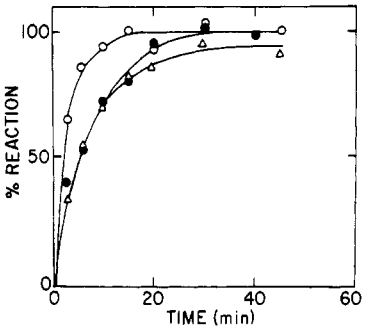

FIGURE 2: Time course of S-sulfonation of the Mcg light chain (Bence-Jones) dimer in the presence of Nbs_2 under three sets of conditions: (○) 100 mM Na_2SO_3 and 1 mM Nbs_2; (●) 50 mM Na_2SO_3 and 1 mM Nbs_2; and (△) 100 mM Na_2SO_3 and 0.2 mM Nbs_2. The kinetics were determined by incorporation of [^{35}S]sulfite into the protein. At specified intervals aliquots were precipitated with 10% trichloroacetic acid. The precipitates were collected and redissolved, and the radioactivity was measured in a scintillation counter.

was measured with a Packard Tri-Carb scintillation counter. The results for the two aliquots were averaged, and the concentrations of free and bound ligand were calculated from the counts per minute and the specific radioactivity of the derivative.

The data were first interpreted by Scatchard analysis with a computerized method (Scatchard et al., 1950; Fletcher & Spector, 1968). Since the Scatchard model is inappropriate for multiple interacting binding sites, the data were also examined by the stepwise equilibrium method (Klotz et al., 1946; Klotz & Hunston, 1979; Fletcher, 1977). A computer program for application of the latter method was kindly provided by Dr. J. E. Fletcher (see Fletcher et al., 1970).

Cleavage and Reoxidation of the Interchain Disulfide Bonds of the Hybrids. After bis(dinitrophenyl)lysine ligands were bound to Mcg covalent dimers and subsequently removed by dialysis, the protein showed markedly different aromatic CD spectra and crystallization properties (Firca et al., 1978; Ely et al., 1978). The conformational changes underlying such observations could be reversed by cleavage and reoxidation of the interchain disulfide bond. This procedure was applied to some samples of hybrids, particularly those used in binding studies and crystallization experiments.

Attempts To Crystallize Hybrids. Initial attempts were based on methods used to crystallize the Mcg Bence-Jones dimer (Edmundson et al., 1972). Samples of hybridized protein in 0.02 M sodium phosphate, pH 7.4, were concentrated to 23 mg/mL by ultrafiltration. Crystallization experiments were conducted at 19 °C and pH 6.2 in 1.3–2.1 M ammonium sulfate which was 0.1 M in sodium phosphate.

Crystallography of the Mcg × Mcg Control "Hybrid". Samples of the S-sulfonated and reduced forms of the protein were mixed to produce an Mcg × Mcg "hybrid". The interchain disulfide bond was cleaved and reoxidized before crystallization of the hybrid in ammonium sulfate. Crystals were subjected to X-ray analysis at 6.5-Å resolution.

Results

Noncovalent Hybridization. Chromatographic fractionation of an Mcg × May hybridization mixture on DEAE-Sephadex A-50 is illustrated in Figure 1. The components were identified by electrophoresis at pH 9.5. The observed order of elution of the May covalent dimer and the more positively charged May MEA derivative was surprising. At present we have no satisfactory explanation for this aberrant behavior, especially since the remaining components emerged in the expected order.

The noncovalent hybrid, appearing in 15% yield as a shoulder on the peak for the Mcg protein, was present in quantities too small for binding and crystallographic studies. The May × Hud noncovalent hybrid was produced in only 5% yield. These low yields are consistent with earlier work by Stevenson & Straus (1968).

S-Sulfonation of the Mcg Protein. Before we adopted the present method of S-sulfonation, alternative procedures were tested for yields and effects on the crystal properties of the derivative. Oxidative sulfitolysis in the presence of Cu^{2+} (Swan, 1957) was quantitative, but the derivative crystallized only in an aberrant needle form in ammonium sulfate (see Ely et al., 1978). Derivatization was slow in the presence of SO_3^{2-} alone, with about 40 h required for completion. Crystals of the derivative included both aberrant needles and trigonal forms. The latter were morphologically similar but not identical with those produced by the native Bence-Jones dimer.

Sulfitolysis in the presence of catalytic amounts of MEA (Chan, 1968) proved to be a mild procedure, but the yields of derivative were only 20–25% (as estimated from the incorporation of $^{35}SO_3^{2-}$). In another mild procedure (Butts & Hedrick, 1969; Vanaman & Stark, 1970), the interchain disulfide was reduced to thiol and reacted with an excess of Nbs_2 to form a protein–Nbs mixed disulfide. Subsequent treatment with sulfite led to liberation of Nbs anions and S-sulfonation of the cysteine residues. The yield of the derivative was about 50%.

In the method preferred for the present study, the addition of catalytic amounts (millimolar) of Nbs_2 led to a marked acceleration of the reaction, relative to trials with SO_3^{2-} alone. Under optimum conditions the reaction was complete in 15 min (see Figure 2). Approximately 1 mol of sulfite was incorporated into each mol of monomer. As a noncovalent dimer, the derivative was electrophoretically homogeneous at pH 9.5 under nondissociating conditions. In the absence of 2-mercaptoethanol but under dissociating conditions in Na-DodSO$_4$ gels, the derivative migrated as a single band in the monomer position. Its electrophoretic behavior was clearly distinguishable from control samples in which the intrachain disulfide bonds were broken with 2-mercaptoethanol. These results suggest that the reaction was confined to one disulfide,

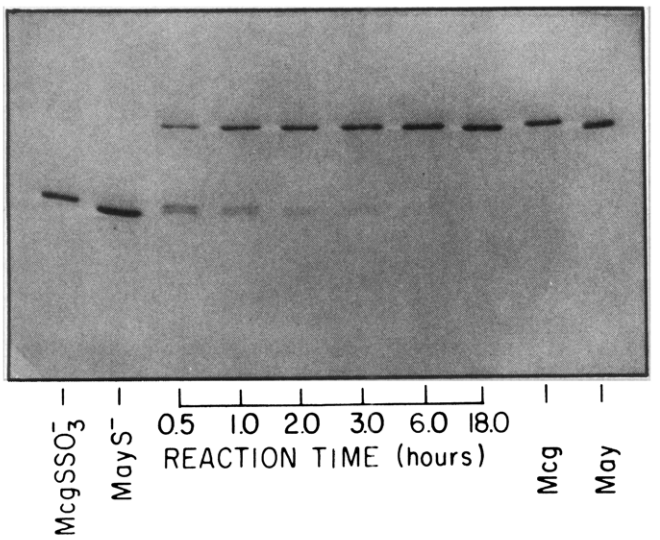

FIGURE 3: Time course of the covalent hybridization of the Mcg and May proteins. The reaction was monitored by $NaDodSO_4$ electrophoresis in the absence of 2-mercaptoethanol by the method of Laemmli (1970). The starting positions correspond approximately to the upper border of the figure. The anode is at the bottom. The banding patterns for the starting samples are shown on the left: the S-sulfo derivative is labeled $McgSSO_3^-$ and the reduced protein is marked $MayS^-$. At the times indicated, aliquots were removed from the mixture, and the reaction was stopped by alkylation of the $MayS^-$ derivative with *N*-ethylmaleimide. Note the appearance of covalent hybrid bands in positions similar to those of the Mcg and May covalent dimer controls.

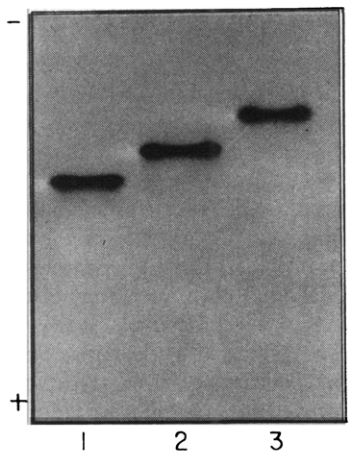

FIGURE 4: Characterization of the Mcg × May covalent hybrid by electrophoresis in the pH 9.5 gel system of Davis (1964). The starting positions are located at the top of the figure. The three samples are (1) the native Mcg covalent dimer; (2) the reaction mixture after covalent hybridization of the Mcg and May proteins; and (3) the native May covalent dimer. Note the absence of the parental species in lane 2.

most likely the accessible interchain bond.

Trigonal crystals of the derivative were obtained in ammonium sulfate. Although the crystals were too small for detailed X-ray analysis, the space group and unit cell dimensions were found to be the same as those for the native Bence-Jones dimer: $P3_121$, with a = 72.3 Å and c = 186.3 Å.

Obligatory Covalent Hybridization. Electrophoretic analyses of the hybridization mixture for the Mcg and May proteins are shown in Figures 3–5. The time course of the reaction is illustrated in Figure 3. $NaDodSO_4$ gel electrophoresis in the absence of 2-mercaptoethanol indicated that a covalent light chain dimer was produced at the expense of the two parental species (designated $McgSSO_3^-$ and $MayS^-$

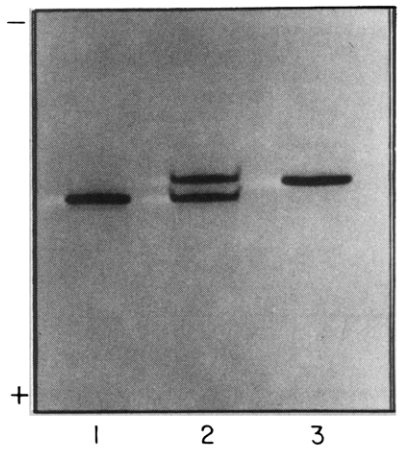

FIGURE 5: Electrophoresis of the Mcg × May hybrid in the acid–urea system of Panyim & Chalkley (1969). The starting positions correspond to the top of the figure. The interchain disulfide bonds were reduced with 2-mercaptoethanol before application of the proteins to the gel. The three samples are (1) the Mcg protein, (2) the Mcg × May hybrid, and (3) the May protein. As expected, the hybrid consisted of equal quantities of the two parental species.

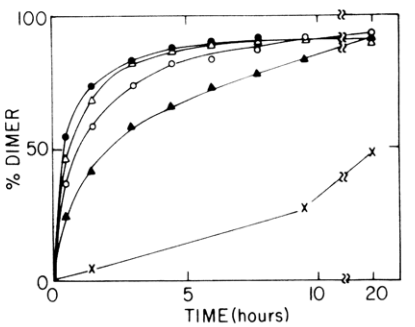

FIGURE 6: Effects of different concentrations of $SrCl_2$ (a sulfite trap) on the apparent rate of covalent hybridization of the Mcg and May proteins. (●) 0.20 M $SrCl_2$; (△) 0.15 M; (○) 0.10 M; (▲) 0.05 M; and (×) 0.0 M. The reactions were followed by $NaDodSO_4$ gel electrophoresis of aliquots taken at the intervals indicated by points on the curves. The fractional hybrid content at each point was estimated from densitometer scans of the stained gels.

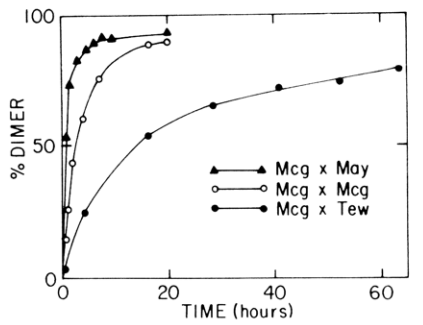

FIGURE 7: The kinetics of covalent hybridizations of three samples, including the Mcg × Mcg control reaction. The Mcg × May (λ × λ) hybridization was one of the faster reactions tested, and the Mcg × Tew (λ × κ) hybridization was the slowest (see Table I). The reactions were followed by $NaDodSO_4$ gel electrophoresis in the absence of 2-mercaptoethanol.

in Figure 3). This dimer, present in a yield >95% after 18 h, was shown to have a mobility intermediate to those of the parental dimers at pH 9.5 (Figure 4). After we reduced the interchain disulfide bond, electrophoresis in acid–urea demonstrated that the produce consisted of equal quantities of Mcg and May light chains (Figure 5).

The effects of a sulfite trap on the apparent rate of covalent hybridization of the Mcg and May proteins are presented in Figure 6. The presence of Sr^{2+} increased this apparent rate

Table I: Yields and Rate Constants of Covalent Hybridizations

hybrid	antigenic classes	yield (%)	second-order rate constants (M^{-1} s^{-1})
Mcg × Mcg	λ × λ	90	2.7
Mcg × Vil	λ × λ	93	2.2
Mcg × Weir	λ × λ	92	1.8
Mcg × May	λ × λ	92	7.9
Mcg × Hud	λ × λ	94	9.8
Mcg × All	λ × λ	95	8.7
Mcg × Bla	λ × λ	97	18.0
Mcg × Cot	λ × λ	90	n.d.[a]
Mcg × Ste	λ × λ	90	n.d.
Mcg × Joy	λ × λ	90	n.d.
Mcg × Tew	λ × κ	78	0.4

[a] n.d., not determined.

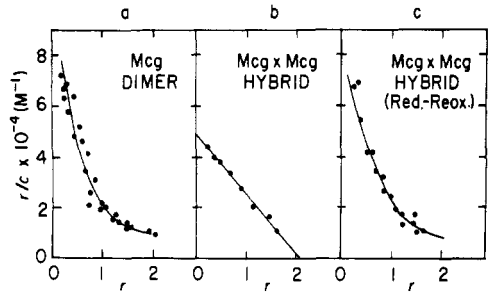

FIGURE 8: Scatchard plots for the binding of bis(dinitrophenyl)lysine by (a) the native Mcg covalent dimer, (b) the Mcg × Mcg control hybrid, and (c) the Mcg × Mcg control hybrid after reduction and reoxidation of the interchain disulfide bond. The curves and line represent the best fits to the data (see text for details).

up to a concentration of 0.2 M.

The kinetics of hybridization for the Mcg and May proteins, the Mcg × Mcg control, and the Mcg and Tew proteins are illustrated in Figure 7. The reaction of the Mcg and May λ chains was typical of rapid hybridizations, while the reaction of the Mcg λ chain and the Tew κ chain was relatively slow. Analyses of the data indicated second-order kinetics.

In the series of hybrids listed in Table I, the second-order rate constants ranged from 0.4 (Mcg × Tew) to 18 M^{-1} s^{-1} (Mcg × Bla). Except for the λ × κ (Mcg × Tew) molecule, the yields of covalent hybrids were ≥90%.

Binding of Bis(dinitrophenyl)[^{3}H]lysine. Scatchard plots for the binding of bis(dinitrophenyl)lysine by the Mcg × Mcg, Mcg × Vil, and Mcg × Weir hybrids are shown in Figures 8–10, with plots for the parent homodimers included for comparison. The Scatchard parameters and stepwise equilibrium constants for these hybrids plus the Mcg × Tew protein are given in Table II.

The plots for the binding by the Mcg (Figure 8a) and Vil (Figure 9a) parent homodimers showed significant curvature. With the curve-fitting program, the data were interpreted in terms of one strong binding site (association constant ~10^5 M^{-1}) and weaker binding sites (constants of 10^3–10^4 M^{-1}). The stepwise equilibrium method gave comparable results. Nonlinear curves were best described by models incorporating three stepwise equilibrium constants. With the low level of binding, however, the k_3 constants were questionable (and are not listed in Table II).

Linear Scatchard plots, indicating two equivalent sites with average association constants of 2–4 × 10^4 M^{-1}, were obtained in the binding studies of the Mcg × Mcg control (Figure 8b) and the Mcg × Vil hybrid (Figure 9b). After the interchain disulfide bonds of these molecules were reduced and reoxidized, however, the plots for ligand binding showed definite curvature (Figures 8c and 9c).

Table II: Binding of Bis(dinitrophenyl)lysine by Selected Light Chain Dimers and Covalent Hybrids

protein	Scatchard parameters[a]	stepwise equilibrium constants[b]
Mcg dimer	$N_1 = 1, N_2 = 4$	
	$K_1 = 1.3 \times 10^5$ M^{-1}	$k_1 = 8.9 \times 10^4$ M^{-1}
	$K_2 = 2.0 \times 10^3$ M^{-1}	$k_2 = 2.6 \times 10^3$ M^{-1}
Mcg × Mcg hybrid	$N = 2.1$	
	$K = 2.3 \times 10^4$ M^{-1}	
Mcg × Mcg hybrid (red.-reox.)[c]	$N_1 = 1, N_2 = 3$	
	$K_1 = 7.3 \times 10^4$ M^{-1}	$k_1 = 8.0 \times 10^4$ M^{-1}
	$K_2 = 2.0 \times 10^3$ M^{-1}	$k_2 = 5.5 \times 10^3$ M^{-1}
Vil dimer	$N_1 = 1, N_2 = 3$	
	$K_1 = 8.2 \times 10^4$ M^{-1}	$k_1 = 9.8 \times 10^4$ M^{-1}
	$K_2 = 1.7 \times 10^3$ M^{-1}	$k_2 = 2.2 \times 10^3$ M^{-1}
Mcg × Vil hybrid	$N = 2.0$	
	$K = 4.1 \times 10^4$ M^{-1}	
Mcg × Vil hybrid (red.-reox.)[c]	$N_1 = 1, N_2 = 2$	
	$K_1 = 9.8 \times 10^4$ M^{-1}	$k_1 = 1.0 \times 10^5$ M^{-1}
	$K_2 = 2.5 \times 10^3$ M^{-1}	$k_2 = 4.7 \times 10^3$ M^{-1}
Weir dimer	no measurable binding	
Mcg × Weir hybrid	$N_1 = 1, N_2 = 2$	
	$K_1 = 1.9 \times 10^5$ M^{-1}	$k_1 = 7.8 \times 10^4$ M^{-1}
	$K_2 = 4.2 \times 10^3$ M^{-1}	$k_2 = 6.2 \times 10^2$ M^{-1}
Tew dimer	$N = 2.4$	
	$K = 2.0 \times 10^4$ M^{-1}	
Mcg × Tew hybrid	$N_1 = 0.35, N_2 = 2.5$	
	$K_1 = 2.1 \times 10^5$ M^{-1}	$k_1 = 3.9 \times 10^4$ M^{-1}
	$K_2 = 3.2 \times 10^3$ M^{-1}	$k_2 = 8.4 \times 10^1$ M^{-1}

[a] N_i is the number of binding sites in each dimer, and K_i is the association constant. [b] The best fit to the data was given in terms of three equilibrium constants, but only two of these are listed. [c] These samples were tested for binding after reductive cleavage and reoxidation of the interchain disulfide bond.

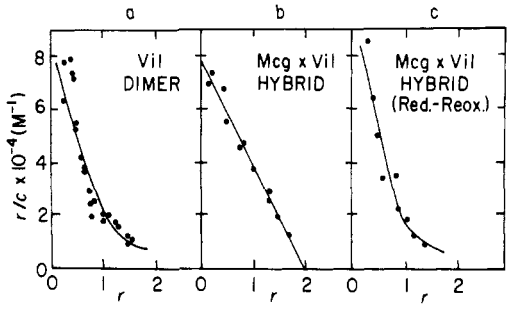

FIGURE 9: Scatchard plots for the binding of bis(dinitrophenyl)lysine by (a) the native Vil covalent dimer, (b) the Mcg × Vil covalent hybrid, and (c) the Mcg × Vil hybrid after reduction and reoxidation of the interchain disulfide bond.

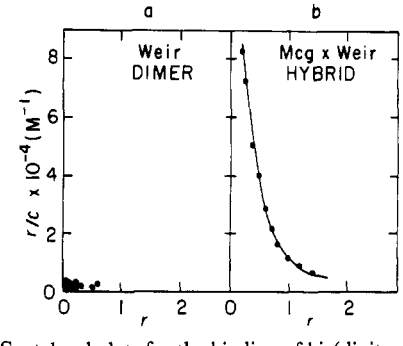

FIGURE 10: Scatchard plots for the binding of bis(dinitrophenyl)lysine by (a) the native Weir covalent dimer and (b) the Mcg × Weir covalent hybrid.

Surprisingly, the Weir homodimer did not bind bis(dinitrophenyl)lysine, while the Mcg × Weir hybrid closely resembled the parental Mcg dimer in binding properties (com-

pare Figure 10 with Figure 8a).

A linear plot, with an average association constant of 2×10^4 M^{-1} for equivalent sites, was produced in the binding of ligand by the Tew κ-type homodimer. A plot with curvature was obtained when the Mcg × Tew hybrid (chromatographically purified) was used in the binding study.

Crystals of Hybrids. Small crystals of Mcg × Bla, Mcg × Vil, Mcg × Hud, and Mcg × Weir hybrids have been obtained in ammonium sulfate. Crystals of the Mcg × Hud and Mcg × Weir hybrids will soon be sufficiently large for preliminary diffraction studies.

Crystallographic Analysis of the Mcg × Mcg Control Hybrid. Crystals of the Mcg × Mcg protein were morphologically indistinguishable from the trigonal form of the native Mcg Bence-Jones dimer. The space group ($P3_121$) and cell dimensions (a = 72.3 and c = 186.6 Å) were also the same as those for the native protein. A comparison by difference Fourier analysis at 6.5-Å resolution revealed that the structures of the two molecules were identical within the errors of the method.

Dissociation of Hybrids after Cleavage of the Interchain Disulfide Bond. In general, the hybrids most reluctant to form (e.g., the Mcg × Tew and Mcg × Weir proteins; see Table II) were also those which most readily disengaged after cleavage of the interchain bond. At the extremes the Mcg × Ste hybrid dissociated to the extent of 85–90% in 24 h, while only half the Mcg × May molecules reverted to the parental species in 2 weeks. In 24 h the dissociation was 40% in the Mcg × Tew and Mcg × Weir hybrids, but <10% in the Mcg × May and Mcg × Hud proteins.

Discussion

Noncovalent Hybridization. Noncovalent dimers exist in a monomer–dimer equilibrium, with typical association constants in the range of 10^4–10^6 M^{-1} (Stevenson & Dorrington, 1970; Björk & Tanford, 1971; Painter et al., 1972; Stevenson, 1973; Green, 1973; Azuma et al., 1974, 1978; Azuma & Hamaguchi, 1976). The limited success in producing noncovalent hybrids adds to the evidence that homodimers are generally the preferred species in a mixture of λ-type light chains. However, there are substantial quantities of stable monomers in some equilibrium mixtures, particularly in those containing κ chains (Kishida et al., 1975; Friedman et al., 1978). The tendency of one of the reactants to remain monomeric would be antagonistic to the formation of noncovalent hybrid dimers. Moreover, even a purified noncovalent hybrid is expected to revert to an equilibrium mixture similar to the one from which it was formed. It was possible to improve the procedure by converting the noncovalent hybrids into stable covalent dimers before fractionation of the equilibrium mixtures, but the approach was abandoned in favor of efficient obligatory hybridization.

S-Sulfonation of the Mcg Light Chain. Crystallographic requirements made it imperative to use mild conditions for S-sulfonation. Side reactions complicating previous applications of S-sulfonation have included modification of tryptophan or losses of enzymatic activity (Bailey & Cole, 1959; Chan, 1968). It is possible that the adverse effects are partly attributable to trace amounts of bisulfite, which is known to modify tryptophan (Morihara & Nagami, 1969).

The appearance of the abnormal needle form in crystallization mixtures of the Mcg protein exposed to sulfite was taken as a sensitive indicator of side effects. Needle formation influenced the decisions to avoid prolonged exposure times or the use of strong oxidizing agents in S-sulfonation. In previous studies (Firca et al., 1978), dissolved needles containing either noncovalent or covalent Mcg dimers were found to have aromatic circular dichroic (CD) spectra markedly different from those of the native dimer. The differences were interpreted in terms of local changes involving tryptophan and tyrosine residues. It was encouraging that the rapid method finally chosen for S-sulfonation of the Mcg protein was relatively free of such adverse side reactions.

Covalent Hybridization. Covalent light chain hybrids can be considered as mixed disulfides synthesized from S-sulfo and thiol derivatives (see Swan, 1957). The general reaction has been applied in the recombination of the A and B chains of insulin (Katsoyannis et al., 1967a,b; Wilson et al., 1962), the artificial conjugation of fragment A of diphtheria toxin with human placental lactogen (Chang & Neville, 1977), and the combination of the component of human chorionic gonadotropin specific for cell surfaces with the toxic subunit of ricin (Oeltmann & Heath, 1979). The yields of these products were lower than those of the light chain hybrids, which have structures highly favorable for this type of reaction.

The high yields of the hybrids had the practical advantage of making purification unnecessary in most cases. These yields were dependent on the presence of a Sr^{2+} trap for the sulfite released during the hybridization reaction (the sulfite would cleave the newly formed interchain disulfide bond). The crystallographic analysis of the Mcg × Mcg control hybrid revealed no major effects of the Sr^{2+} on the three-dimensional structure of the protein.

While the rates of hybridization vary widely (see Table I), it is clear that all light chains tested to date hybridize with the Mcg protein. The method appears to be sufficiently general for other light chains to work as well.

Possible Mechanism of Covalent Hybridization. We favor a mechanism invoking a monomer–dimer equilibrium of the parental species. If two noncovalent homodimers are mixed, heterodimers can be produced by subunit exchange. Orienting noncovalent forces then bring the $-SSO_3^-$ and $-S^-$ groups into sufficiently close proximity to form an interchain disulfide bond. The hybridization is thereby rendered irreversible.

This mechanism is supported by several lines of evidence. As stated earlier, the existence of monomer–dimer equilibria is well documented. The favorable role of noncovalent orienting forces is suggested by an experiment demonstrating that covalent hybridization is inefficient in 5 M urea. Kishida et al. (1976) previously reported that urea inhibited covalent dimerization of light chains. The sizable differences in the kinetics of hybridization of the Mcg protein with various λ chains are not easily reconciled with alternative mechanisms involving random collisions of $-SSO_3^-$ and $-S^-$ groups. The C domain sequences of λ chains are similar, with the Mcg protein differing from other molecules in no more than five positions (Fett & Deutsch, 1976). Since these residues are spatially removed from the interchain disulfide bond (see Ely et al., 1978), the immediate environments of the $-SSO_3^-$ and $-S^-$ groups should be the same in the hybridization mixtures.

The differences in kinetics were strong arguments for the rate-limiting step to be the association of parental monomers into heterodimers. This conclusion is consistent with the results of Azuma et al. (1974), who reported that differences in V domains are mainly responsible for the diversity in equilibrium constants for dimerization of light chains.

Dissociation of Hybrids after Cleavage of the Interchain Disulfide Bond. The differences in the V domains probably also account for the wide variation in the tendency of the hybrids to dissociate after cleavage of the interchain bond. This explanation seems particularly appropriate for the relative

instability of the Mcg × Weir hybrid. The C-domain sequences of the parental species differ only by a lysine–glutamic acid (Mcg–Weir) interchange at position 160 (Fett & Deutsch, 1976). In contrast, the V domains belong to different subgroups (V for Mcg and II for Weir). In the partially known sequence of the Weir V domain, 4 of the first 22 residues are different from those of the Mcg protein.

The ease of dissociation of the Mcg × Tew hybrid was predictable. There are extensive differences in the sequences of λ and κ chains, and dissociation is also expected to be promoted by the tendency of the Tew protein to exist in a stable monomeric form.

The marked tenacity with which the Mcg and May (or Mcg and Hud) λ chains remained together was surprising, particularly in view of the low yields in noncovalent Mcg × May hybridization attempts. We concluded that during the hybridization process one or both subunits participated in conformational changes resulting in higher noncovalent affinity for each other.

Binding of Bis(dinitrophenyl)lysine by Hybrids. Both the Scatchard and stepwise equilibrium methods indicate that the native Mcg Bence-Jones dimer has one strong binding site and weaker sites for the bis(dinitrophenyl) ligand. An asymmetrical pattern of binding was also found in the crystal (Edmundson et al., 1974). The crystallographic results can be briefly summarized with the aid of a perspective drawing of the binding regions, with the sites designated by letters (see Figure 11). Bis(dinitrophenyl)lysine was found by difference Fourier analysis to bridge sites A and B, with one Dnp ring in site A of monomer 2 and the second in site B. Site A′ of monomer 1 was lined by side chains homologous to those of monomer 2. However, access to site A′ was blocked by crystal-packing interactions, and ligands even smaller than bis(dinitrophenyl)lysine were not admitted. Bis(dinitrophenyl)lysine began to appear in the deep pocket (site C) shortly before the crystal disintegrated. The ligand could only appear in site C by forced entry and local expansion of the pocket.

In solution, the most probable binding sites are therefore tandem sites A and B, plus A′ and a putative B′. Inspection of the atomic model of the native dimer shows that a second Dnp ring cannot be accommodated unless the binding cavity is expanded in the vicinity of site B. We were therefore happy to accept the concept of asymmetrical binding in the native dimer in solution.

Immediately after hybridization, however, the Scatchard analyses of the Mcg × Mcg and Mcg × Vil proteins clearly indicated symmetrical binding of bis(dinitrophenyl)lysine in two equivalent sites. The expected asymmetrical binding patterns appeared only after cleavage and reoxidation of the interchain disulfide bond. These findings suggest that the formation of this bond during hybridization preceded and prevented final noncovalent conformational adjustments like those occurring in dimerization of the parental species. The binding cavity was thus locked into a symmetrical conformation with respect to its ability to bind two ligands. The ease with which the symmetrical and asymmetrical species can be interconverted emphasizes the inherent flexibility in and around the binding sites of these molecules.

The Mcg × Weir hybrid is particularly interesting since the Weir parental dimer does not bind the Dnp ligand. This is an example of a hybrid molecule in which the binding specificity is dependent on the amino acid sequence of one subunit in a geometric setting provided by the interactions of two dissimilar chains.

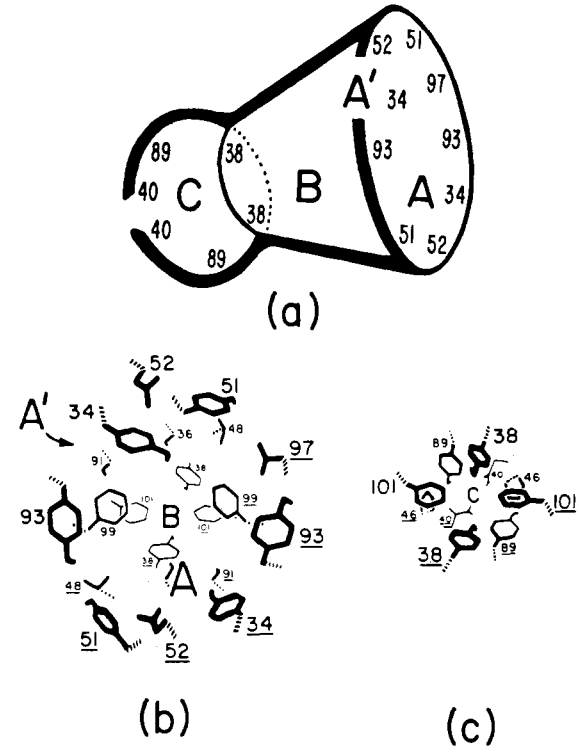

FIGURE 11: Schematic views of the binding regions for dinitrophenyl compounds, as delineated in previous crystallographic studies of the Mcg Bence-Jones dimer (Edmundson et al., 1974). (a) Side view. Key side chains are numbered in appropriate positions. Bis(dinitrophenyl)lysine bridged sites A and B, with one dinitrophenyl ring in each site. The ligand was also detected in site C, but caused the crystal to disintegrate when the occupancy exceeded about 0.2 mol/mol of dimer. Access to site A′ was blocked by packing interactions in the crystal, but should be available for ligand binding in solution. (b) Perspective drawing of the side chains lining the main binding cavity containing sites A, A′, and B. The residue numbers of monomer 2 constituents are underlined. The α–β carbon bonds are represented as dotted lines. (c) Side chains lining the deep pocket, reached through the floor of the main cavity and containing site C.

Correlation of Dnp Binding with the Tendency of Light Chains To Form Amyloid Fibrils. Bertram et al. (1980) recently found that amyloidogenic light chains in a series of 13 samples bound bis(dinitrophenyl)lysine while the control samples did not. The Mcg and Tew Bence-Jones proteins were both obtained from patients with amyloidosis (Deutsch, 1971; Osserman, 1976), and it is significant that both parental dimers and the hybrid showed similar binding behavior with bis(dinitrophenyl)lysine. Amyloidogenic light chains usually have greater affinities for tissue components than those from patients without amyloidosis. It is generally felt that these tissue affinities are related to the binding regions of the light chains. If the Mcg dimer is taken as a prototype of an amyloidogenic protein, we have a three-dimensional model for a typical binding region of such a protein (see Figure 11). Despite the fact that the V domain sequences of the Mcg and Tew proteins differ in 64 positions (Fett & Deutsch, 1974; Putnam et al., 1973; Osserman, 1976), the crucial contact residues for Dnp binding are present in both molecules (tyrosine-34 and -38 and phenylalanine-101 in Figure 11). In position 99 (Mcg numbering), threonine in the Tew protein replaces phenylalanine, a substitution which should increase the space for binding in site B.

As predicted by Kabat et al. (1977), the similarities in the V domain sequences of the Mcg and Vil proteins were reflected in the binding properties of the parent molecules and the Mcg × Vil hybrid. Again, the only substitution in the binding sites

involves residue 99, in which valine (Vil) is substituted for phenylalanine (Ponstingl & Hilschmann, 1969).

Concluding Remarks

A procedure was developed for obligatory covalent hybridization of similar or dissimilar immunoglobulin light chains. All samples tested formed hybrids with the Mcg λ chain in high yields. The properties making the Mcg protein so favorable for structural research were generally passed on to the hybrids. For example, the hybrids crystallized with greater frequency than the non-Mcg parental dimer. The binding properties of the hybrids were qualitatively and quantitatively similar to those of the Mcg dimers. In one case (Weir), a nonbinder was coupled to an Mcg monomer to produce a hybrid with the desired specificity for bis(dinitrophenyl)lysine.

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