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Covalent Immunoglobulin Assembly in vitro: Reactivity of Light Chain Covalent Dimers (L_2) and Blocked Light Chain Monomers

Abstract. *Covalent light chain dimers (L_2) and cysteine-blocked L chain monomers readily react with partially reduced heavy (H) chains. A rapid disappearance of these blocked L chain species is followed by the appearance of covalent intermediates—HL, H_2, and H_2L—leading to fully assembled H_2L_2. The mechanism of initial disulfide bond formation between heavy and light chains is disulfide interchange.*

In a study of the factors controlling antibody assembly, we have investigated in vitro the covalent assembly of a human IgG1κ immunoglobulin (Fro) (*1*). The quaternary structure of proteins of this immunoglobulin class is maintained by strong noncovalent forces as well as by four covalent interchain disulfide bonds. Two of these link the heavy (H) chains to each other, and two bridge heavy to light (L) chains, utilizing the carboxy terminal light chain residues and residue 220 in each heavy chain (*2*).

The interchain disulfides can be reduced without detectable reduction of any of the intrachain bonds (*3, 4*). Our experimental procedure, accordingly, entails selective (partial) reduction of the four interchain disulfide bonds, followed in certain experiments by reoxidation without prior disruption of the noncovalently associated tetramer (*3*) and in others by reoxidation after the H and L chains are separated and then recombined in varying molar ratios (*4*). The last type of experiment permits study of the assembly and reoxidation processes when L chains are modified, or present in excess, as is often the case both in normal lymphoid cells (*5, 6*) and in mouse myeloma tumors or cloned cell lines (*7*).

Methods have been devised for the quantitative determination of assembly intermediates during reduction as well as reoxidation (*3, 8*), so that not only initial and final states, but the kinetics of conversion from one to the other can be analyzed. Moreover, a general theory has been formulated to compare assembly in vitro with reported intracellular pathways and kinetics of assembly (*9*).

Among the key experimental findings with the IgG1κ studied are (i) that the rates and pathways of covalent assembly are not significantly affected by whether or not the H and L chains are separated from one another prior to reoxidation at neutral *p*H, and (ii) that large molar excesses of L over H chains do not markedly affect the pathways compared to equimolar reoxidations, although excesses diminish the rate of covalent H_2L_2 assembly. At the same time, excess L chains retard the formation of insoluble H chain aggregates in vitro, and this suggests a comparable role for such excesses in vivo (*4*).

The theoretical analysis revealed that the reoxidation process is not random; the four interchain disulfide bonds form with unequal probability throughout the course of the reaction. Initially, HL bonds form about twice as rapidly as HH bonds, but the formation of the second HL bond in the molecule is not favored over HH bond formation. In other words, the existence of one HL bond in a noncovalently assembled tetramer slows the rate at which the second forms.

Preliminary results have also established that covalent L_2 dimers rapidly react with reduced H chains or H_2 dimers and generate intermediates that assemble normally into H_2L_2 tetramers, a finding unanticipated by any previous studies on immunoglobulins (*10, 11*). Our report further documents this result and demonstrates also the reaction of partially reduced H chains with a stable L chain monomer whose carboxy terminal cysteine residue is in mixed disulfide linkage with the amino acid L-cysteine (*12*).

These L chain forms are derived from the urinary Bence Jones protein. Chromatography on Sephadex G-100 yields two fractions, a covalent L chain dimer and a stable L chain monomer. These forms are not interconvertible, and neither contains detectable free sulfhydryl (*12*). Procedures for preparation and purification of the plasma monoclonal protein (Fro) and of the partially reduced H chains have been published (*3, 4*), as have all of the other analytical procedures employed in this study (*3, 4, 11*). For recombination experiments, reduced H chains and unreduced dimeric or

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blocked monomeric L chains were mixed under nitrogen in equimolar quantities at *p*H 3.2, in 10 m*M* acetic acid. Reoxidation conditions were established by exposing the mixture to air, and raising the *p*H to 7.5 and the ionic strength to 0.14. At various times during the reoxidation, portions were removed and reacted with excess iodoacetamide. These alkylated samples were then subjected to electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). Reactants (H,L,L_2), product (H_2L_2), and the three covalent intermediates—HL, H_2, H_2L—were identified by their migration distances relative to the unreduced protein and partially reduced H and L chains (*3*, *4*).

Figure 1 shows the gels from an experiment in which partially reduced H chains were reacted with the Bence Jones covalent dimer containing a small amount (less than 10 percent) of blocked L monomer (well 2). By the first reoxidation time point (well 3) L_2 disappeared and intermediates leading to H_2L_2 were observed, with noticeable increases in L, HL, and H_2L. Control experiments, aimed at eliminating the possibility that thiols of low molecular weight were involved, established that the isolated L_2 fraction, when maintained under reaction conditions for 1 hour or longer, ran true on gels and molecular sieve columns, as well as in sedimentation experiments. Moreover, Gally and Edelman (*10*), in an early investigation of L chain dimers, showed that alkylated heavy chains are not reactive with L_2.

These intermediates, therefore, arise by two disulfide interchange reactions:

$$H + L_2 \rightarrow HL + L \quad (1)$$

$$H_2 + L_2 \rightarrow H_2L + L \quad (2)$$

After these initial interchange reactions, the reoxidation closely resembles other experiments with unblocked, reduced L chain preparations. Quantitative densitometry of the gels (*4*) showed that the pathways are comparable, as inferred from the maximum intensities achieved by the intermediates, and from the positions of gel band maxima on an abscissa representing the average number of disulfide bonds formed at any point during the reoxidation (*3*). In addition, the kinetics of the assembly process agree with those in other 1:1 recombinations, as judged by the half-times of sulfhydryl disappearance.

In other experiments with covalent L_2 dimers, the H chain fraction was largely in the form of H_2. In such cases, reaction 2 predominates, as indicated by a high initial level of H_2L.

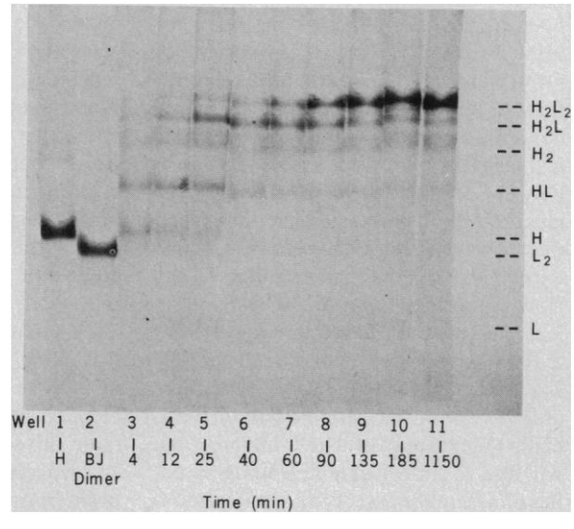
Partially reduced H chain was also combined with the Bence Jones mixed disulfide monomer, as shown in Fig. 2. At the first time point (well 4) there was an increase in HL, in accord with release of cysteine from the mixed disulfide:

$$H + L\text{-}S\text{-}S\text{-cysteine} \rightarrow HL + \text{cysteine} \quad (3)$$

Again, gel analysis revealed that once HL is formed, the assembly proceeds with the same pathways. A distinctive feature of this reoxidation, however, is that at the final time point (well 11) the H_2L level is still high. Moreover, the rate of sulfhydryl disappearance is about 20 percent slower than in experiments using free, reduced L chain.

Intracellular covalent L_2 dimers are observed in mouse myeloma tumors which synthesize excess L chains (*5*). Although it has been argued that L_2 is unlikely to be an assembly intermediate (*13*), the present experiments demonstrate that these forms of L chain, as well as monomers in mixed disulfide linkage with cysteine (or, possibly, other low-molecular-weight thiols), are reactive with H chain or H_2 dimers, and lead to productive intermediates for covalent tetramer assembly. Furthermore, the initial interchange reactions are rapid compared to the remaining covalent bond formations and do not depress the overall assembly rates, except in the case of the blocked monomer. Kazin (*12*) has shown that the interchange reactions actually occur in at least two stages. An initial rapid reaction results in the disappearance of about one-half of L_2 dimer or blocked L monomer within 1.5 minutes. A second stage leading to 90 percent or greater disappearance requires 30 to 40 minutes for the dimer and as long as 90 minutes for the blocked L chain monomer.

More detailed studies of both noncovalent and covalent aspects of these interactions are required before it can be decided what role disulfide interchange may play in the intracellular assembly process. There have been suggestions



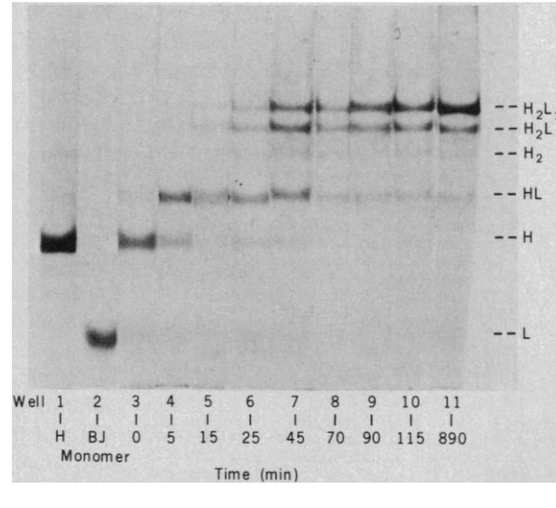


Fig. 1 (top). The reoxidation of partially reduced heavy (*H*) chain with light chain covalent dimer (L_2) shown on SDS-polyacrylamide gel. Wells 1 and 2 represent the purified H chain and Bence Jones (*BJ*) covalent L chain dimer, respectively, after Bio-Gel P2 chromatography. The H and L_2 fractions were combined under N_2 at an equimolar ratio in 10 m*M* acetic acid (final ratio of total L chain to H chain = 1.36). The *p*H of the mixture was then raised to 7.5 and the mixture was exposed to air. The final concentration of H_2L_2 was 1.6 μM. Wells 3 to 11 represent portions from the reoxidation mixture which were immediately alkylated with 1.0*M* iodoacetamide at the times indicated. All gel samples contained 1 percent SDS and iodoacetamide. Fig. 2 (bottom). The reoxidation of partially reduced H chain with cysteine-blocked light chain monomer (*L*) shown on SDS-polyacrylamide gel. Wells 1 and 2 represent the purified H chain and Bence Jones monomer fractions which were combined at an equimolar ratio as in Fig. 1. However, here the final ratio of L chain to H chain was 1.14, and the concentration of H_2L_2 was 2.0 μM. Well 3 represents a portion from the mixture that was alkylated just prior to the *p*H being raised above 7.5. Wells 4 to 11 represent reoxidation samples that were alkylated at the times indicated.

that enzymes may be involved in intracellular disulfide interchange or in catalyzing formation of correct disulfide bonds in immunoglobulins, but there is no conclusive evidence of such intracellular activity (*14*). It seems clear, however, that the blocked L forms discussed in this work, and possibly a variety of other nonproductive intermediates in assembly, can be readily converted to molecular species on the main pathways without enzymatic participation. The apparently small kinetic barriers thus may allow thermodynamic control over the final covalent assembly steps.

In this last connection, the blocked L monomer experiment is interesting and exceptional in that an incompletely assembled intermediate, H_2L, remains in high excess after very long times. A possible explanation is that the released cysteine forms a stable mixed disulfide with H_2L. It is more likely, however, that H_2L is not as reactive as H with L-cysteine. This provides an analogy with the reoxidation system using free L chains in which, as noted above, the second HL bond is formed more slowly than the first. In this view, the driving force for these reactions is the establishment of forms which yield maximal concentrations of tetramer, even if not all disulfide bonds are formed within the tetramer, and the last bond to form, in this case between H_2L and L, may add only marginal stability. The somewhat diminished rate of sulfhydryl disappearance in this experiment is then probably due to the slow rate of oxidation of the cysteine released in reaction 3.

It is hoped that experiments of this kind, coupled with studies of incomplete assembly with the variable half of the L chain (V_L) instead of whole L chain (*15*), will lead to a more complete understanding of assembly defects and may also allow controlled preparation of various intra- and interspecies hybrids.

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DNA Synthesis in Cartilage Cells Is Stimulated by Oscillating Electric Fields

Abstract. *External oscillating electric fields (1166 volts per centimeter, 5 hertz) enhanced the incorporation of [^{3}H]thymidine into the DNA of chondrocytes isolated from the proliferative layer of embryonic (16 days) chick epiphysis. Verapamil or tetrodotoxin at 10^{-6}M concentrations completely blocked the electric field effect. Tetracaine reduced the incorporation of [^{3}H]thymidine in both control and electrically stimulated cells. The findings support the hypothesis that Na^+ and Ca^{2+} fluxes generated by the electrical perturbation trigger DNA synthesis in these cells.*

Electrical stimuli, applied in various ways, promote appositional and longitudinal bone growth in birds (*1*), limb regeneration in amphibia (*2*), and fracture repair in dogs, rabbits, and humans (*3*). Electric fields (EF's) stimulate proline uptake in cultured fibroblasts (*4*) and cause dedifferentiation in amphibian erythrocytes (*5*). Investigation of the mechanism of these effects can contribute to the understanding of epigenetic control of growth and differentiation. In this study we report that oscillating EF's stimulate the incorporation of [^{3}H]-thymidine into the DNA of cartilage cells in suspension, and that this effect can be blocked by inhibitors of calcium or sodium fluxes.

Cartilage cells were isolated from the proliferative zone of tibia epiphyses from 16-day chick embryos (*6*). The cells were incubated for 6 hours in a defined medium at 37°C in the presence of [^{3}H]thymidine and were exposed to a pulsed d-c external EF of 1166 volt/cm, oscillating at 5 hertz. The EF was chosen on the basis of its inhibitory effect on adenosine 3′,5′-monophosphate (cyclic AMP) accumulation (*7*), a condition conducive to cell proliferation in certain systems (*8*). Under these conditions the EF invariably stimulated [^{3}H]thymidine incorporation, by 53 ± 13 percent (mean ± standard error, 72 experiments, 216 samples and matched controls, range: 24 to 120 percent). The EF-enhanced incorporation of [^{3}H]thymidine occurred in material that could be digested with deoxyribonuclease, whereas the labeling of the material that could be extracted with trichloroacetic acid was unaffected. The EF effect was first detected after 6 hours of incubation. The lag period suggests that the EF acts on a regulatory step in the cell cycle which precedes DNA synthesis. We also found an increase in DNA synthesis 4 to 6 hours after a brief electrical stimulation (15 minutes).

Skin fibroblasts obtained by collagenase digestion from 10-day-old chick embryos and treated in the same way as the chondrocytes were not stimulated by the EF. Under the same experimental conditions, the EF failed to affect [^{3}H]thymidine incorporation into lymphocytes from rat spleens. However, in bone cells isolated from calvaria of 19-day rat embryos (*9*). EF's enhanced [^{3}H]-thymidine incorporation by 27 ± 1.2 percent (three experiments, 15 samples). Therefore, under the experimental conditions used, the effect appears to be tissue specific.

In formulating a hypothesis for the mechanism of this phenomenon, we took into consideration the similarity of the effects of mechanical and electrical stimuli on bone growth (*1, 3*) and on cellular cyclic AMP (*6, 7, 10*) and current theories on the control of cell proliferation (*11*). Previously, we showed that mechanical stimuli capable of influencing bone growth reduced cellular cyclic AMP in epiphyseal chondrocytes through enhancement of calcium uptake and inhibition of adenyl cyclase (*7*). Modulation of ion fluxes and cyclic AMP levels are assumed (*12*) to be general modes of membrane-mediated cellular communication, which reached evolu-

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