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THE EFFECT OF CUPRIC ION ON THE REOXIDATION PATHWAY OF AN IgG1 IMMUNOGLOBULIN*

Mollie N. Pflumm, Fred. K. Friedman, Eva Fischer and Sherman Beychok†

Department of Biological Sciences and Chemistry, Columbia University New York, New York 10027 and NIADDK National Institutes of Health, Building 10, Room 9N-307, Bethesda, Maryland 20205

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ABSTRACT: The addition of a 5-10μM of cupric ion to the reoxidation medium of a partially reduced IgG protein results in an altered pathway for covalent reassembly of the molecule. The formation of HL is favored over that of H_2 and H_2L.

Immunoglobulins, as multi-chain proteins, require a unique quarternary structure for activity. Their molecular assembly is a complex process involving at least three steps: first, individual chains acquire a defined structure, possibly their native one. The folded subunits then associate noncovalently. Then subsequently, the interchain disulfide bonds form.

In the IgG class, two disulfide bonded heavy-light chain pairs are joined by two or more interheavy chain S-S bridges. There are three possible pathways for H_2L_2 assembly and six possible intermediates, which might appear during the process (1,2). However, no single pathway gives rise to all the intermediates and, since all six are seen during the in vitro reoxidation of reduced IgG, multiple pathways exist. We have recently observed that the presence of Cu^{+2} in the reoxidation medium enhances the formation of HL over other intermediates and thus the addition of cupric ion provides a means of isolating and studying the HL pathway of immunoglobulin assembly.

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†Author to whom all correspondence should be addressed.

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Cupric ions and other divalent metal ions exert a well known catalytic effect on the oxidation of sulfhydryl groups to disulfides by molecular oxygen (3). One mechanism proposed for thiol oxidation involves catalysis by trace metals (4,5). In numerous kinetic investigations, IgG assembly has yielded varying half-times in reoxidation which might be explained by the presence of Cu^{+2} in the reoxidation medium (6-8). Cupric ions have been shown to play a role in the regeneration of reduced lysozyme (9) and, in conjunction with o-phenanthroline, in the oxidation of SH groups in aldolase (10). While lysozyme is a single-chain protein which requires denaturation for reduction and undergoes great conformational changes during reoxidation, the interchain disulfide bonds of immunoglobulins may be reduced under non-denaturing conditions. Moreover, the occurrence of the sulfhydryl residues in close proximity to each other in an area rich in proline (11) provides steric constraints (12) which make it likely that the formation of covalent IgG intermediates involves minimal refolding with the oxidation of thiol residues. Since methods for the quantification of the various immunoglobulin intermediates are available (13) we studied the effect of adding divalent copper (in the form of $CuCl_2$) on IgG assembly.

The effect of cupric ion on disulfide formation is also important since this pairing of cysteine residues has often been used as a probe in studying protein folding (14-16). The sulfhydryl containing residues are easily identified and their oxidation may be readily blocked at any time by the addition of an alkylating agent. Comprehensive examinations of the role of S-S bond formation in the refolding pathways of bovine pancreatic trypsin inhibitor (17, 18) and ribonuclease A (19) and a study of the role of disulfide bridges in globular proteins (20) have recently appeared. However, the results of our studies indicate that, at least in the case of IgG, the presence of Cu^{+2} alters the pathway of reoxidation. This indicates that extreme caution must be used in removing or accounting for divalent ions when probing folding pathways with S-S reoxidations.

MATERIALS AND METHODS

The protein used for this study (Fro) was isolated from the plasma of a patient suffering from asymptomatic plasma cell dyscrasia (21) by methods pre-

viously described (1). IgG (Fro) comprises γ1 heavy chains and κ light chains (Osserman, unpublished results). The plasma was generously given to us by Professor Osserman.

Protein concentrations were determined using an extinction coefficient of 1.91×10^5 M^{-1} at 278 nm (22). The protein (4-5 mg/ml) was dissolved in 0.15M Tris-HCl, 0.15M NaCl, 2mM EDTA, pH 8.1 and its interchain disulfide bonds reduced by incubation with a 200-300 fold excess of dithiothreitol (DTT; Calbiochem 233155) for 60 min. at room temperature under a stream of nitrogen. Excess DTT and buffer salts were removed by gel filtration on Bio-Gel P-2 (Bio-Rad) using nitrogen-saturated 10mM acetic acid as the eluant. The concentration of the nitrogen-saturated reduced protein solution was determined spectrophotometrically on a Cary 15 recording spectrophotometer and the solution aliquoted into six vials. Sulfhydryl reoxidation was initiated by the addition of one-tenth volume 0.5 M Tris-HCl, 1.0 M NaCl and the appropriate concentrations of Cu^{+2} to yield buffer compositions of 0.05 M Tris-HCl, 0.1 M NaCl, 0.009 M acetate, pH 7.5 containing 0, 1μM, 2μM, 5μM or 50μM $CuCl_2$. The protein was allowed to reoxidize in open vials at room temperature. At appropriate times, 450μl aliquots of the reassociating proteins were removed and added to separate vials containing 50μl 1M recrystallized iodacetamide (Sigma I-6125) to block further covalent assembly. Following the addition of 55μl 10% SDS (Bio-Rad, electrophoresis grade) and boiling, the samples were subjected to quantitative polyacrylamide gel electrophoresis using an ORTEC Model 4200 electrophoresis system as described earlier (13). Two slight modifications were imposed on the earlier procedure: All the samples were boiled 3-4 minutes prior to electrophoresis and the gels were stained overnight in 0.02% Coomassie brilliant blue (Schwarz/Mann), then destained for several days at room temperature. It was found that the dilute staining procedure minimized background. The partially assembled IgG intermediates are quantified in terms of fractional moles, a quantity defined as the observed amount of a given species divided by the maximum amount of that species which could be generated during the reoxidation (1, 22). Thus all species are listed as quantities between 0 and 100%.

RESULTS AND DISCUSSION

In the absence of copper, the molecular reassembly follows the pattern shown in Figure 1a. The amount of each molecular species is shown as it varies with time. Characteristically, first HL, then H_2 and H_2L reach their maximum value. However, the magnitude of the H_2L maximum is greater than that of H_2.

The influence of Cu^{+2} on the course of reassembly was studied by adding 1, 2, 5, 10 and 50 μM Cu^{+2} to the reoxidation medium. Figure 1b shows the curves obtained in the presence of 50 M Cu^{+2}. As seen in Figure 2, increasing the concentration of Cu^{+2} leads to a decrease in the amount of free H and L chains left after 21 min. reaction time with concomitant increases in the amounts of H_2L_2 and HL generated.

To determine the effect of the divalent cation on the reaction pathway, we have examined the distribution of the assembly intermediates at a fixed point in the reassembly process. Figure 3 shows the results obtained when the fractional moles of H_2L_2 is 40%. The most prominent feature of this graph

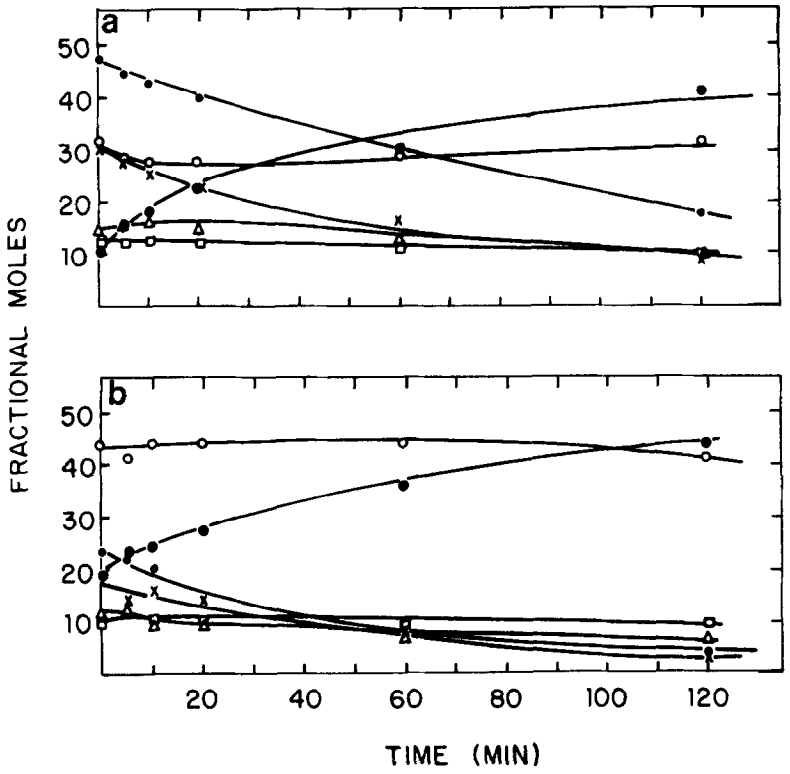

Figure 1a. The reoxidation of IgG (Fro) in the absence of cupric ion. The molecule contains γ heavy and κ light chains. The protein was allowed to reoxidize at a concentration of 4.6μM in 0.05 Tris, 0.1 M NaCl, 9mM acetate, pH 7.5 at room temperature. The course of the reaction was followed for two hours. (·), L; (x), H; (o), HL; (□), H_2; (Δ), H_2L; (●), H_2L_2.

Figure 1b. The same reoxidation as it occurs in the presence of 50μM Cu^{+2}.

is the marked increase in HL which is accompanied by a drop in the amounts of H_2L and L.

Although the catalytic effect of divalent cations on the air oxidation of thiols is long established (3), protein reoxidations appear more complex. High levels of Cu^{+2} have been shown to inhibit the regeneration of native taka-amylase A (23) and lysozyme (9) and the presence of large amounts of Cu^{+2} was shown to affect the reoxidation pathway of IgG4 immunoglobulins (8). It has been proposed that the high Cu^{+2} levels result in such rapid disulfide formation that no free SH is left to catalyze further interchange, thus trapping incorrect bonds. While this explanation may apply to the single-chain proteins, it is not likely to suffice in the case of IgG where the molecule's constituent

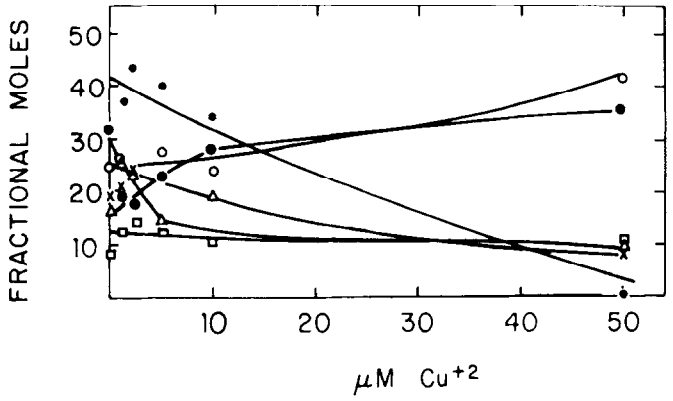

Figure 2. The effect of Cu^{+2} on the reoxidation of IgG (Fro) observed after 21 min. of reaction at room temperature. The protein was allowed to reoxidize at a concentration of 4.6μM in 0.05 M Tris, 0.1 M NaCl, 9mM acetate, pH 7.5 in separate vials containing 0, 1, 2, 5, 10 or 50μM cupric ion. (·), L; (x), H; (o), HL; (□), H_2; (Δ), H_2L; (•), H_2L_2.

chains are already assembled non-covalently and possess little freedom to form incorrect bonds.

In the data we have presented, Cu^{+2} appears to alter the pathway of IgG reassembly. Quite clearly, the rates of formation of all types of S-S bonds are not affected equally by the divalent metal. Although there is an initial surge in H_2L_2 formation, the presence of copper clearly favors the accumulation of HL. There is no enhancement in the production of L_2, H_2 or H_2L and, after

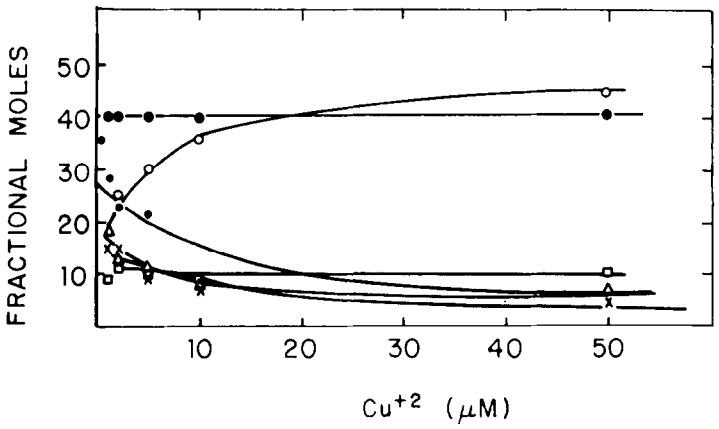

Figure 3. The effect of Cu^{+2} concentration on the distribution of IgG assembly intermediates when the reoxidation is 40% complete. The reaction was allowed to proceed under the conditions described for figure 3 and data was obtained from the curves in figure 2. (·), L; (x), H; (o), HL; (□), H_2; (Δ), H_2L, (•), H_2L_2.

long reoxidation times (22½ hours, unpublished observations), less H_2L_2 forms when Cu^{+2} is present than when it is not. The relatively large amounts of Cu^{+2} required to produce the observed effects, 5-10μM when the concentration of reoxidizing protein is 4.6μM, suggest the possibility that a nonproductive complex forms between Cu^{+2} and some protein thiols. In any event, the addition of large amounts of Cu^{+2} provides a means of preparing IgG half molecules, HL, which may be useful in future studies.

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