Monoclonal Antibody Refolding and Assembly: Protein Disulfide Isomerase Reaction Kinetics

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The protein disulfide isomerase (PDI) reaction kinetics has been studied to evaluate its effect on the monoclonal antibody (MAb) refolding and assembly which accompanies disulfide bond formation. The MAb *in vitro* assembly experiments showed that the assembly rate of heavy and light chains can be greatly enhanced in the presence of PDI as compared to the rate of assembly obtained by the air-oxidation. The reassembly patterns of MAb intermediates were identical for both with and without PDI, suggesting that the PDI does not determine the MAb assembly pathway, but rather facilitates the rate of MAb assembly by promoting PDI catalyzed disulfide bond formation. The effect of growth rate on PDI activities for MAb production has also been examined by using continuous culture system. The specific MAb productivity of hybridoma cells decreased as the growth rate increased. However, PDI activities were nearly constant for a wide range of growth rates except very high growth rate, indicating that no direct correlation between PDI activity and specific MAb productivity exists.

Key words: monoclonal antibody, protein folding and assembly, protein disulfide isomerase, continuous culture, hybridoma

INTRODUCTION

The endoplasmic reticulum (ER) is the site where not only the secretory and lysosomal proteins, resident luminal ER proteins, Golgi, and lysosomal membrane proteins are synthesized, but most extensive folding and oligomeric assembly take place [1, 2]. Since most secretory and membrane glycoproteins contain disulfide bonds, formation and rearrangement of disulfide bonds are critically important for efficient transport and sorting of newly synthesized proteins. Unfolded and unassembled polypeptides are retained because they form aggregates [3] and they are unable to enter transport vesicles or because they are bound to the resident ER proteins such as Bip [4]. Misfolded and misassembled polypeptides are also retained in the ER and degraded in ER itself [5].

The protein disulfide isomerase (PDI) is one of the key resident ER proteins that are essential for disulfide bond formation in the cell [6, 7]. Recently, considerable effort has been devoted to improve understanding of the catalytic properties of PDI [8-14] and multiple roles in the modification of secretory proteins [3, 15, 16]. In large part, the formation of disulfide bonds has been studied for further understanding of the chemistry of protein folding and oligomeric assembly. But very limited studies have been concerned with the PDI catalyzed reaction kinetics for monoclonal antibody (MAb) refolding and assembly.

In this study, our goals are to elucidate the reaction kinetics of PDI catalyzed MAb refolding and assembly in the ER and to develop a strategy to improve MAb productivity. The analysis of the reaction kinetics of PDI catalyzed MAb refolding and assembly *in vitro* and *in vivo* should provide us with some insight into the role of PDI in the ER and, perhaps, enable us to develop improved methods for refolding and assembly of other proteins.

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MATERIALS AND METHODS

Cell Line and Medium

A mouse-mouse hybridoma cell line, 66'F11, producing IgG_1 against cephalexin-synthesizing enzyme was used in this study [17]. The cells were derived by fusing mouse myeloma (P3-X63-Ag8.653) cells with female Balb/c mice spleen lymphocytes immunized with purified cephalexin-synthesizing enzyme (CES). Cells were maintained at 37°C in a 5.5% CO_2-in-air humidified incubator by using the RPMI-1640 medium (RPMI, Gibco Laboratories, N.Y.) containing 10% serum (HS:FBS=9:1, Gibco). The medium was supplemented by 100 units/mL penicillin-G and 100 mg/mL streptomycin (Gibco) in 150 cm^2 tissue culture flasks (T-flasks). The initial glucose concentration in the medium was 2.0 g/L.

Preparation of PDI and MAb

PDI can be readily solubilized from microsomal membranes and purified from several higher eukaryotic sources. In this study the purified PDI was prepared from bovine liver microsomes, which involves homogenization in Triton, heat treatment at 54°C, selective ammonium sulfate precipitation (55-85%) and cation

(CM-sephadex) and anion (DEAE-sephacel) exchange chromatography. Further details of the preparative procedures and the characterization of the enzyme are given by Hilson and Lambert [18, 19]. We have also prepared MAb by using cell line 66'F11 secreting IgG_1 against cephalexin synthesizing enzyme (CSE). The protocols for cell culture conditions, large-scale perfusion culture of hybridomas, and MAb purification methods are described in elsewhere [17, 20, 21].

In Vitro MAb Refolding and Assembly

To study the reaction kinetics of PDI catalyzed MAb refolding and assembly an *in vitro* system [22] was employed. Using 10 mM dithiothreitol (DTT), the purified IgG was first reduced and the heavy (H) and light (L) chains were dialysed against 1.0 M acetic acid. After noncovalent recombination of chains under anaerobic conditions at slightly acidic pH by dialysis against 4 mM sodium acetate buffer (pH 4.8), the chains were reassembled in the presence of protein disulfide isomerase. The samples were withdrawn at various times and the disulfide bond formation was terminated by alkylation with 10 to 20 mM iodoacetamide (IAA). The alkylated disulfide intermediates during MAb assembly reaction were resolved by running SDS-PAGE under nonreducing condition (0.01% bromophenol blue, 4% SDS, 60 mM IAA, and 20% glycerol in 25 mM Tris buffer, pH 8.2). The relative intensity of the stained protein bands was then measured at 595 nm (coomassie blue) using a spectrodensitometer (SD3000, Kratos). The concentration of each protein component was then calculated by using standard calibration curve obtained from several known concentrations of intact IgG, H chain, and L chain.

PDI Activity During Continuous Culture

The cells were cultivated pseudo-continuously in 8 different T-flasks in a 6% CO_2/air incubator at 37°C. The cells were seeded at about 1×10^5 cells per mL. The continuous culture was started after 3-day cultivation of seed. The culture working volume was maintained as 200 mL and fresh medium (RPMI 1640) containing 10% serum (HS:FBS=9:1) was added twice daily. The dilution rates were varied from 0.2 to 0.9 per day. In the range of dilution rates (0.2-0.9 day^{-1}), a reasonably stable culture was maintained and all steady-states were characterized by constant concentrations of cell, glutamine and antibody. For the assays, 10 mL of the culture samples were taken twice daily and cells were separated by centrifugation at 600 g for 5 min and washed three times with PBS buffer. The supernatant was frozen at -80°C for later determination of MAb. The remaining cells were stored at -80°C for PDI activity assay.

Thawed cell pellets were homogenized with a tight Teflon/glass homogenizer (1200 rev./min, 50 strokes) in a cold solution of 0.5 mL lysis buffer (0.2 M NaCl, 0.1% Triton X-100, 0.05 M Tris/HCl buffer, pH adjusted to 7.5 at 4°C. The homogenates were centrifuged at 15000 g for 30 min at 4°C), and aliquots of the supernatants were used immediately for the assays. PDI activity was assayed by the modified procedure of Lambert and Freedman [23], in which the reactivation of scrambled RNAase (SRNAase) by PDI is monitored by a spectrophotometric assay of RNAase activity. Further details are described elsewhere [9].

RESULTS AND DISCUSSION

Fig. 1(a) shows the control experiment of MAb assembly in the absence of PDI. In the early stage, heavy (H) chain is consumed faster than light (L) chain and subsequent assembly occurs through a defined pathway involving distinctive intermediates (H_2, HL, and H_2L). The light chain dimer (L_2) was not detectable, indicating that thiol groups forming disulfide bonds between L chains are far less reactive than those of other intermediates. The overall assembly reaction was almost completed within 30 hrs. The yield of almost 3 μM H_2L_2 obtained after 33 h incubation represents about 75% of the theoretical maximum (i.e., 4 μM).

The reaction pattern of MAb reassembly facilitated by PDI was basically identical to that without PDI in terms of the intermediates and their relative levels of maximum concentrations (Fig. 1(b)). But, the H_2L_2 production rate with PDI was markedly faster than that without PDI. The mean-half time's of two assembly reactions were 100 and 900 min, respectively. Clearly, these results illustrate two important points. First, shorter mean half-time indicates that PDI greatly accelerates MAb assembly rate. The observed difference between the slow MAb assembly in the absence of PDI and the rapid MAb assembly in the presence of PDI suggests that the MAb assembly process is enzyme-catalyzed. The productivity of MAb can be improved by cloning the PDI gene and increasing the level of PDI enzyme, if folding and MAb assembly in the ER are rate limiting. Second, our finding that the reaction mechanisms and reaction kinetics for both PDI catalyzed and air-oxidized MAb assembly are qualitatively the same, suggests that PDI does not affect the assembly reaction mechanism or pathway leading to the formation of H_2L_2. Some interconversions between MAb assembly intermediates, which normally are not significant, seem to occur in the presence of PDI.

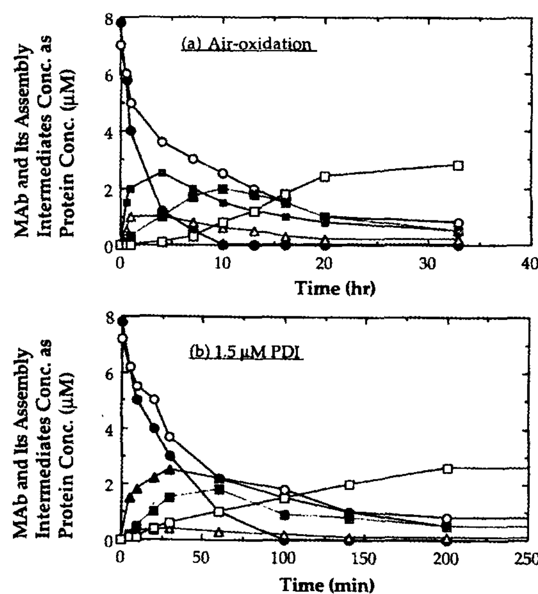

Fig. 1. Kinetics of MAb assembly *in vitro*; (●) heavy chain (H), (○) light chain (L), (▲) HL intermediate, (△) H_2 intermediate, (■) H_2L intermediate, (□) assembled H_2L_2; (a) in the absence of PDI and (b) in the presence of PDI.

Since immunoglobulin G (IgG) is composed of two H and two L chains linked by disulfide bonds, two H-H and two H-L, a new reaction mechanism is proposed and depicted in Fig. 2. Based on *in vitro* MAb assembly experiment, it was found that three principal intermediates (H_2, HL, H_2L_2) were involved and the formation of light chain dimers (L_2) was negligible (see Fig. 1).

The rate expression for the MAb assembly can then be written as:

$$\frac{d[L]}{dt} = -k_2[H][L] - k_4[H_2][L] - k_6[H_2L][L]$$

$$\frac{d[H]}{dt} = -k_1[H]^2 - k_2[H][L] - k_3[HL][H]$$

$$\frac{d[HL]}{dt} = k_2[H][L] - k_3[HL][H] - k_5[HL]_2$$

$$\frac{d[H_2]}{dt} = k_1[H]^2 - k_4[H_2][L]$$

$$\frac{d[H_2L]}{dt} = k_3[HL][H] + k_4[H_2][L] - k_6[H_2L][L]$$

$$\frac{d[H_2L_2]}{dt} = k_6[H_2L][L] + k_5[HL]^2$$

The rates of formation of disulfide bond between intermediates were assumed to follow simple first-order reaction kinetics. Based on kinetic data obtained from *in vitro* assembly experiment, the rate constants of assembly reactions k_1, k_2, k_3, k_4, k_5, and k_6 were evaluated by using the method of steepest descent [24] after solving the fitting equations by numerical integration, and the results are presented in Table 1. There are two types of disulfide bonds in MAb assembly, namely, between H and L and between H and H. As can be seen from Table 1, a greater discrepancy in the reactivities of the -SH groups formimg the two types of disulfide bonds was apparent. Complete assembly of heavy and light chains occurs very rapidly in the presence of PDI, the ratios between rate constants vary from 5 fold to 10 fold. It is also noted that the reaction rates of disulfide bond formation of the same type are different. These studies also suggest that the utilization of particular MAb assembly pathway depends on structural features of the Ig such as the intrinsic reactivity of the -SH groups and the number of disulfide bonds.

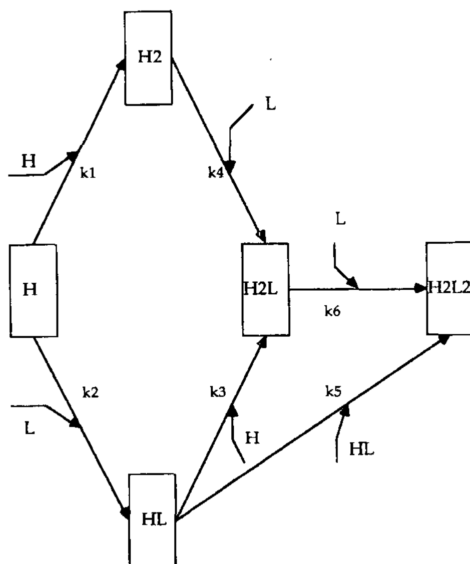

Fig. 2. Reaction mechanism of MAb refolding and assembly.

To examine the effects of growth rate on the levels of PDI activity and MAb productivity in hybridoma cells, continuous culture was employed to control the specific growth rate that has been shown to affect the specific MAb productivity [25-27]. Fig. 3(a) shows the steady-state viable and total cell concentrations at various dilution rates. The viable and total cell concentrations decreased almost linearly for the higher dilution rate range (0.7-0.9 day^{-1}), while they were nearly constant for the lower dilution rate region (0.2-0.6 day^{-1}). The cell viabilitiy in terms of viable cell fraction increased with increasing dilution rate and the maximum viability corresponded to the maximum dilution rate. These trends in cell growth observed are similar to those measured for several other hybridoma cell lines [25, 26].

The specific rates of cell growth and cell death are shown in Fig. 3(b). The specific growth rate was slight-

Table 1. Rate constants of monoclonal antibody refolding and assembly

Rate constants (L/μmole/h)	Air oxidation	with 1.5 μM PDI	Disulfide bonds
k_1	0.010	0.080	H-H
k_2	0.016	0.122	H-L
k_3	0.020	0.146	HL-H
k_4	0.003	0.015	H_2-L
k_5	0.014	0.067	HL-HL
k_6	0.003	0.033	H_2L-L

Refer to Fig. 3 for the designation of rate constants

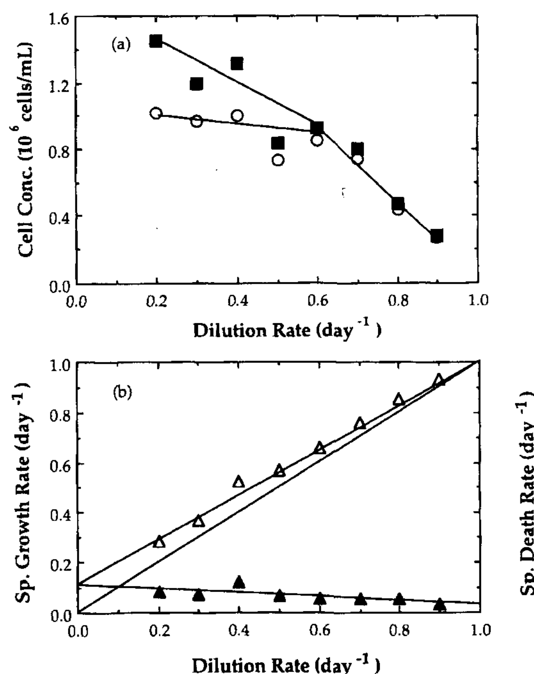

Fig. 3. Effect of dilution rate on hybridoma cell growth; (a) viable cell concentration (○), total cell concentration (■) and (b) specific growth rate (△), specific death rate (▲) under steady-state conditions.

ly greater than the dilution rate (see the diagonal line in Fig. 3(b)) due to the adjustment for the dead cell and it showed a linear relationship in the dilution rate range evaluated, 0.2-0.9 day^{-1}. The specific death rate decreased linearly with increasing dilution rate.

Fig. 4(a) shows the steady state values of PDI activity per cell at different growth rates. It should be noted that the PDI activities increase at higher specific growth rates (0.86-0.93 day^{-1}) while they are practically constant at lower or moderate growth rate range (0.28-0.76 day^{-1}). The specific antibody production rate as a function of the specific growth rate is shown in Fig. 4(b). The steady-state values of the specific MAb productivity increased about 3 fold from 6.1 to 19.2 mg/10^9 cells/day when the growth rate was decreased from 0.86 to 0.28 day^{-1}. It is not clear at present why the PDI activity increases despite decreasing specific antibody productivity (see Fig. 4(b)) at higher specific growth rates. It is plausible that additional PDI requirement for production of other cellular proteins must increase at higher growth rates.

We have shown that the rate of MAb assembly with PDI can be greatly enhanced, but there was no direct relationship between PDI activity and specific antibody productivity during continuous culture of hybridoma cells. The results of this study are very different from finding that the levels of intracellular PDI content greatly vary with the Ig secretory activities of various different mouse spleen and lymphoid cell lines under similar growth conditions [28, 29]. It appears that increase in PDI activity at higher growth rate is due to requirement of other cellular proteins. Higher specific MAb productivity at lower specific growth rate is still controversial and it is not likely that more PDI activity is required due to higher MAb production at

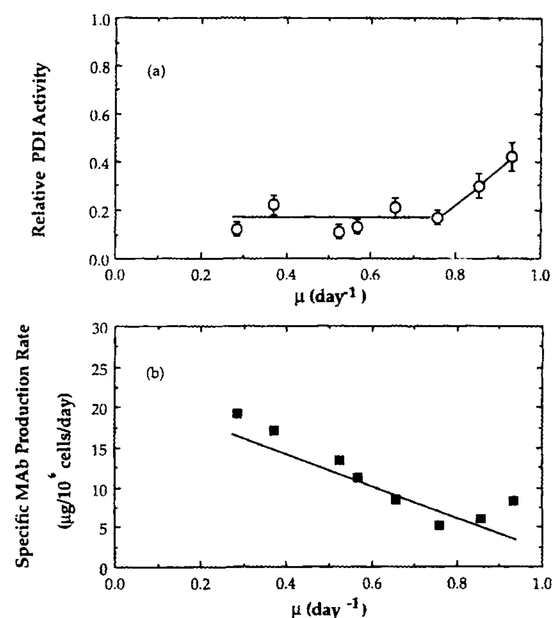

Fig. 4. Effect of growth rate on PDI activity and MAb productivity; (a) relative PDI activity (○) and (b) specific MAb production rate (■). PDI activity is based on 105 cells and three consecutive measurements at steady-state were averaged at each dilution rate. Vertical error bars indicate the deviations.

lower growth rate.

The ER processing has been proposed as a possible rate-limiting step among various post-translational events by showing that different membrane and secretory proteins move from the rough ER to the Golgi at very different rates, and the processing time in the ER is generally longer than those of other intracellular processes [30, 31]. Two plausible explanations for different processing times at ER have been postulated. First, different proteins go through post-translational process in ER at different rates, most likely because different proteins with different composition and three dimensional structure requires different processing time to fold and assemble [2]. Another important reason for the proteins having differential exit rates from ER is the protein transport process from ER to Golgi after folding and assembly of newly synthesized proteins is completed. Our data suggest that MAb refolding and assembly in the endoplasmic reticulum may not be a rate limiting step, but the transport process of MAb from ER to Golgi seems to be critically important in determining the overall rate of the MAb synthesis and secretion process.

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