Secondary Structure of the Murine Histocompatibility Alloantigen H-2K^b: Relationship between Heavy Chain, β_2-Microglobulin, and Antigenic Reactivity†

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ABSTRACT: The far-ultraviolet circular dichroism (CD) spectra of the extracellular portion (papain-cleaved fragment) of the histocompatibility antigen H-2K^b and its noncovalently associated components, heavy chain and β_2-microglobulin (β_2m), indicate that the antigen is highly structured, containing about 30% α-helix, 41% β-sheet, and 29% random coil. Separation of β_2m from the heavy chain produced a decrease in heavy chain α-helix and β-sheet structure which correlated with a loss of alloantigenic reactivity. Reconstitution of the heavy chain–β_2m complex resulted in an increase in secondary structure which was greater than the sum of the free chains and the recovery of considerable alloantigenic reactivity. This suggests that some of the secondary structure and much of the alloantigenic reactivity may depend on conformation associated with the binding of β_2m to heavy chain. A prediction of heavy chain secondary structure based on Chou–Fasman analysis of the primary amino acid sequence agreed with results from CD measurements and suggested that the segments of α-helix and β-sheet structure are distributed throughout the molecule.

The major histocompatibility complex (MHC) antigens of the mouse (H-2 class I) are heterodimers consisting of a heavy chain, polymorphic, transmembrane glycoprotein of M_r 44 000 noncovalently associated with an extracellular protein of M_r 11 600, β_2-microglogulin (β_2m) (Kimball & Coligan, 1983). These H-2 class I alloantigens are expressed by most types of cells. Although they were discovered because they serve as targets for graft rejection in vivo, their biological function may be to associate with foreign antigens for presentation to the cellular immune system. This interaction then leads to the immunological recognition and elimination of cells expressing foreign antigens such as virally infected cells (Zinkernage & Doherty, 1979). The human homologues to the murine H-2 class I antigens are the HLA class I molecules (Strominger et al., 1981; Kimball & Coligan, 1983).

Primary structural studies of the H-2K^b heavy chain, at the protein (Nathenson et al., 1981; Kimball & Coligan, 1983) and DNA level (Weiss et al., 1983), have determined the complete amino acid sequence (348 amino acids) and suggest that H-2 antigens may be divided into five regions. These consist of three extracellular domains of approximately 90 residues each (α1, α2, and α3), a transmembrane region of 24 hydrophobic residues, and a carboxy-terminal, cytoplasmic region of 35 amino acids. Although the intact H-2K^b antigen is insoluble, the extracellular region was first isolated as a water-soluble fragment noncovalently bound to the β_2m subunit by cleavage at residue 281 with the proteolytic enzyme papain (Martinko et al., 1980).

Although the primary structure of H-2K^b is known, there is little information concerning the relationship between secondary and tertiary structure and functional regions. Recent data have indicated that the α1 and α2 domains contain most of the antigenic sites recognized in humoral or cellular allo responses (Nairn et al., 1980; Hämmerling et al., 1982; Sherman, 1982; Clark & Forman, 1983; Ozato et al., 1983; Reiss et al., 1983; Allen et al., 1984) and that β_2m binds to the α3 region (Yokoyama & Nathenson, 1983). In this report we describe some of the secondary structural properties of the extracellular region of the H-2K^b molecule as deduced from circular dichroism (CD) studies and present some features of its secondary structure as suggested by theoretical prediction methods.

Materials and Methods

Antisera. The alloantisera (BALB·G α EL-4) and (BALB·G α BALB/c) that were used to detect the H-2K^b and H-2K^d molecules were prepared as previously described (Ewenstein et al., 1978). The Y-3 hybridoma cell line was a gift from Dr. E. A. Lerner and C. Janeway (Yale University, New Haven, CT). The monoclonal antiserum it produces reacts with the H-2K^b determinant (Jones & Janeway, 1981).

Purification of the H-2K^b Papain Fragment. The H-2K^b fragment, generated through proteolysis by papain, consisting of most of the extracellular portion of the molecule (amino acids 1–281), was used for this study. The methods employed for the purification of the papain-derived product and the separation and isolation of its constitutents, β_2m and heavy-chain papain fragment, have been described in earlier work (Yokoyama & Nathenson, 1983). Briefly, glycoproteins from the lymphoma cell line EL-4 (H-2^b) were isolated from NP-40-solubilized cell extracts by using *Lens culinaris* affinity chromatography. The glycoproteins were digested with papain and the cleavage products separated by gel filtration on Sephadex G-150 and chromatography on DEAE-Sephadex A-25. In each case, the H-2K^b-rich fractions were detected by a standard radio immunoprecipitation assay (Yokoyama & Nathenson, 1983). These fractions were pooled, and the H-2K^b papain fragment was isolated by affinity chromatography on a column of normal rabbit IgG conjugated to Sepharose 4B followed by a column of Y-3 (anti K^b) IgG conjugated to Sepharose 4B.

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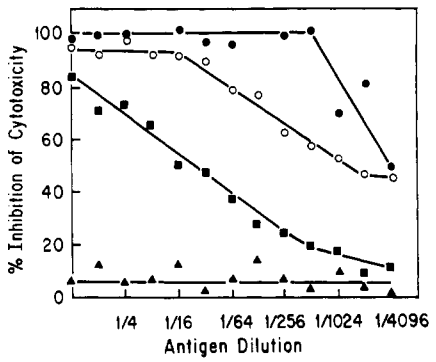

FIGURE 1: Antigenic activity of the H-2K^b papain fragment and its components. Inhibition of complement-mediated anti-H-2K^b alloantisera-dependent cytotoxicity of C57BL/10 (H-2^b) splenic lymphocytes was determined for the -H2K^b papain fragment (●), H-2K^b heavy-chain papain fragment (■), β_2m (▲), and reconstituted H-2K^b papain fragment (○). Initial antigen concentrations were at 50 μg/mL.

To separate the heavy-chain papain fragment from β_2m, the H-2K^b papain fragment was treated with 3 M NaSCN and fractionated by gel filtration on Sephadex G-200 (Kvist et al., 1977). To reconstitute the H-2K^b papain complex, equimolar amounts of heavy-chain fragment and β_2m were incubated together in 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, and 0.15 M NaCl for 4 h at 37 °C and dialyzed against 20 mM Tris, pH 8.0, and 0.15 M NaCl, and the reconstituted complex was isolated by gel filtration on Sephadex G-200.

The antigenic activity of the isolated H-2K^b components was measured by competitive inhibition of anti-H-2K^b or K^d alloantisera in antibody-dependent complement-mediated cytotoxicity of ^{51}Cr-labeled C57BL/6 (H-2^b) and B10·D2 (H-2^d) splenic lymphocytes (Sanderson, 1964).

Circular Dichroism Spectra. Circular dichroism spectra were measured on a Cary Model 60 spectropolarimeter with a Model 6002 CD accessory. Protein concentrations were determined by the method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951). Measurements were taken at 27 °C in cylindrical cells with 1.0-cm path lengths with duplicate experiments of two or more protein concentrations in the range between 0.02 and 0.1%. The slit width was programmed for a spectral band width of 15 Å at all wavelengths, and an absorbance of 2.0 was not exceeded. No concentration effects were observed. The mean residue ellipticities in (deg·cm^2)/dmol and the amounts of α-helix, β-sheet, and random coil were calculated by established methods (Greenfield & Fasman, 1969; Chen et al., 1972, 1974).

Secondary Structural Predictions. The Chou–Fasman model (Chou & Fasman, 1978) for predicting secondary structure based on primary amino acid sequence[1] was used to produce a theoretical structure for the H-2K^b heavy-chain papain fragment for data in Table II and for the H-2K^b heavy chain for plotting Figure 3.

RESULTS

Antigenic Properties of the H-2K^b Fragments. The isolated H-2K^b papain fragment was homogeneous as determined by analysis on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, which showed the expected M_r 37K heavy chain and M_r 12K β_2m, and N-terminal amino acid sequencing (18 amino acids), which showed the H-2K^b sequence consistent with the repetitive yield (data not shown). To determine whether the H-2K^b papain fragment and its component chains retained alloantigenic sites, they were tested for their ability to inhibit the lysis of C57BL/10 (H-2K^b) target cells by an anti-H-2K^b alloserum made in BALB·G mice against the H-2^b cell line EL-4. As shown in Figure 1, the titer at which lysis was inhibited by 50% was 5096 for the H-2K^b papain fragment, 32 for the heavy chain papain fragment, and 1024 for the H-2K^b heavy chain–β_2m reconstituted papain complex. A monoclonal antibody against H-2K^b gave similar results (data not shown). These results indicate that the purified H-2K^b products are antigenically active although the heavy chain is about 0.6% and the reconstituted complex 20% as active as the H-2K^b papain fragment. They did not nonspecifically inhibit the lysis of B10·D2 lymphocytes by alloantisera against H-2K^d as a control (data not shown).

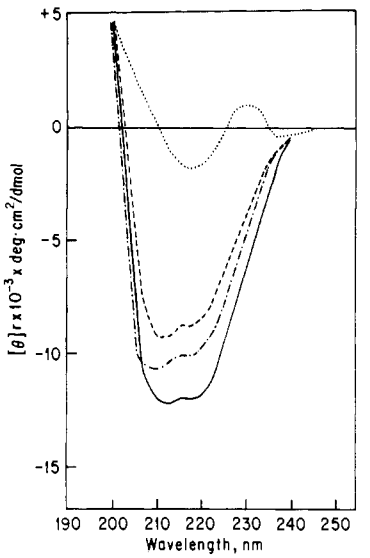

FIGURE 2: Circular dichroic spectra of the H-2K^b papain fragment and its components, expressed as ellipticity per mean residue. H-2K^b papain fragment (—); heavy-chain papain fragment (- - -); β_2m (···); reconstituted H-2K^b papain fragment (- - -).

Table I: Relative Amounts of Secondary Structural Forms in the H-2K^b Papain Fragment and Its Components

protein	measurement	α-helix (%)	β-sheet (%)	random coil (%)
H-2K^b papain fragment	CD	30	41	29
H-2K^b reconstituted papain fragment	CD	20	45	35
free heavy-chain papain fragment	CD	21	30	49
β_2-microglobulin	CD	8	42	50
glycophorin A	CD	23	16	61
	published[a]	27	10	63

[a] Schulte & Marchesi (1979).

CD Spectrum of the H-2K^b Papain Fragment and Its Components. The far-UV CD spectrum, pepide or amide absorption region, of the H-2K^b papain fragment and its component chains is shown in Figure 2. A similar CD spectrum profile was observed for the papain fragment, the isolated heavy-chain papain fragment, and the reconstituted H-2K^b papain complex. These spectra contain two large negative bands, one at 213–215 (214) nm and the other at 218–220 (219) nm. The relative amounts of α-helix, β-sheet, and random coil structure as estimated from the CD spectra are presented in Table I.

The values for the heavy-chain papain fragment secondary structure were obtained both directly from its CD spectrum (observed) (Table I) and indirectly by subtracting the con-

[1] The computer program used for the Chou–Fasman analysis was programmed in Fortran by Dr. Fred Cohen at Yale University.

Table II: Secondary Structure of Free or β_2m-Associated Heavy Chain

protein	measurement	α-helix (%)	β-sheet (%)	random coil (%)
free heavy-chain papain fragment	CD	21	30	49
heavy chain in H-2K^b papain fragment	calculated[a]	39	40	21
heavy chain in reconstituted H-2K^b papain fragment	calculated[a]	25	46	29
free heavy-chain papain fragment	predicted[b]	36	29	35

[a] Calculated values by subtracting the β_2m contribution from that of the H-2K^b papain fragment. [b] Predicted values from Chou–Fasman analysis.

tribution of β_2m from the H-2K^b papain fragment of the reconstituted papain fragment (calculated) (Table II). The rationale for determining the secondary structure of the heavy-chain papain fragment from the H-2K^b papain fragment is based on the assumption that β_2m maintains the same secondary structure whether alone or in association with heavy chain (Brodsky et al., 1974; Isenman et al., 1975). β_2m contributes 28% of the secondary structure in the H-2K^b papain fragment based on protein molecular weight, while the heavy chain papain fragment (M_r 30 000 without carbohydrate) contributes 72% of the secondary structure. The amount of α-helix, β-sheet, or random coil structure attributed to the H-2K^b papain fragment by its components may be calculated by taking 28% of the values for β_2m and 72% of the values for heavy-chain measurements.

The spectrum of β_2m was considerably different from that of the H-2K^b papain fragment and heavy-chain papain fragment, with a small negative band at 219 nm which crossed over to a positive value at 210 nm. This profile is characteristic of a protein with large amounts of β-structure and similar to reports for human β_2m (Karlsson, 1974; Isenman et al., 1975; Lancet et al., 1979; Trägardh et al., 1979).

Glycophorin A was used in this study to evaluate the validity of the CD measurements. As shown in Table I, the amounts of secondary structure observed for glycophorin A agree with previously published values (Schulte & Marchesi, 1979).

Predicted Secondary Structure of the H-2K^b Heavy Chain. A possible model for the secondary structure of the H-2K^b heavy chain is presented in Figure 3. It suggests that the α-helix and β-sheet segments are distributed throughout the molecule. The model predicts 36% α-helix, 29% β-sheet, and 35% random coil structure for the papain fragment of this molecule, values similar to those obtained from the CD spectrum (Table II).

Discussion

The CD analyses reveal that the H-2K^b papain fragment is a highly structured molecule containing 30% α-helix and 41% β-sheet structure (Table I). Our observations suggest that some of the secondary structure and much of the alloantigenic reactivity may depend on conformation associated with the binding of β_2m to heavy chain. This is based on the concomitant decrease in amounts of ordered secondary structure and alloantigenic reactivity in the heavy-chain papain fragment when the β_2m subunit is removed. The reassociation of β_2m with the heavy-chain papain fragment results in a partial restoration of alloantigenic reactivity and also appears to produce an increase in ordered secondary structure, especially β-sheet. This conclusion is based on the finding that 30% of the free heavy-chain papain fragment structure is β-sheet conformation, although 40% β-sheet and 46% β-sheet

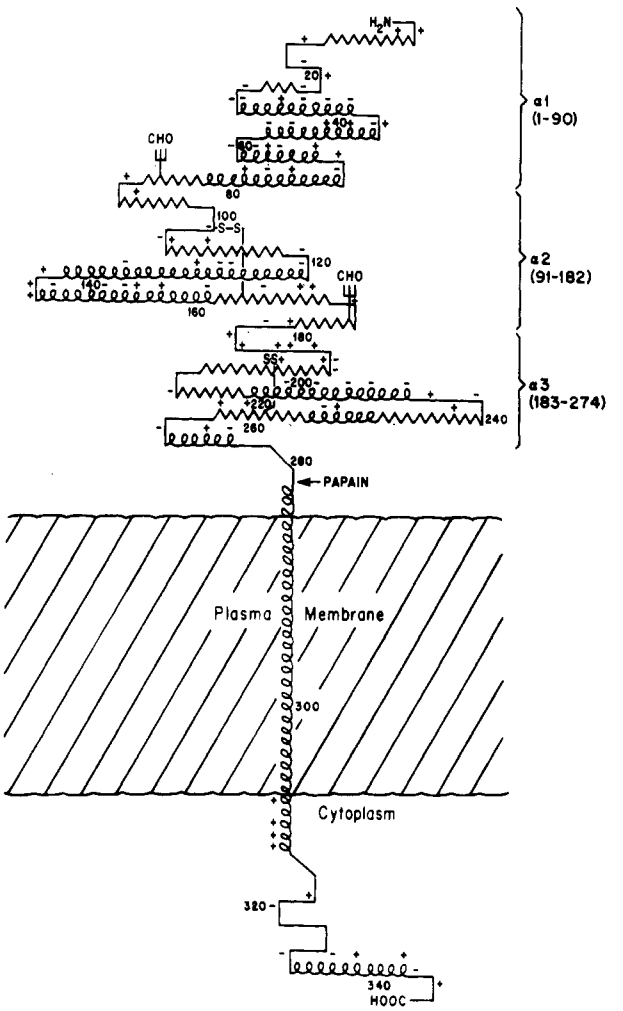

FIGURE 3: Predicted secondary structure of the H-2K^b heavy chain. α-Helix (loops); β-sheet ($\wedge$); β-turn ([); random coil (—). Charged residues are indicated by (+) or (−).

were calculated for the heavy-chain papain fragment in the native and the reconstituted fragment by subtracting the contribution of β_2m (Table II). This difference is probably not due to an irreversible denaturation of the heavy-chain papain fragment during isolation, since there was no appreciable change in β-sheet structure between the whole papain fragment (41% β-sheet) and the reconstituted complex (45% β-sheet) (Table I). In contrast, the significantly greater amounts of α-helix structure calculated for the heavy-chain papain fragment in the native fragment (39%), compared with that of the free heavy-chain papain fragment (21%), may be due to denaturation (unfolding) since it is not recovered in the reconstituted fragment (20%) (Table II). An increase in secondary structure resulting from an interaction between β_2m and heavy chain has also been suggested for HLA molecules (Lancet et al., 1979).

The suggestion that the association of β_2m with heavy chain is necessary to maintain the integrity of alloantigenic sites is supported by the finding of 32-fold greater alloantibody binding activity for the H-2K^b reconstituted fragment when compared with that of the heavy-chain papain fragment alone (Figure 1). However, not all H-2K^b alloantigenic sites may require the presence of β_2m, since free heavy-chain papain fragment alone was weakly active (0.6%).

Although the MHC class I murine and human products are structurally homologous and share 70–80% of their amino acid sequence, our data suggest that they exhibit differences in

secondary structure. The CD spectra for the H-2K^b papain fragment and the heavy-chain papain fragment have two minima (214 and 219 nm); the HLA papain fragments are reported to have only one (between 217 and 219 nm) (Lancet et al., 1979; Trägardh et al., 1979). The H-2K^b papain fragment is 30% α-helix, 41% β-sheet, and 29% random coil (Table I), compared with 8% α-helix, 89% β-sheet, and 3% random coil for the HLA-B7 molecule (Lancet et al., 1979). These results suggest that there are differences in both α-helix and β-sheet content between mouse and human class I antigens. In contrast, the circular dichroism spectrum for murine β_2m (Figure 2) is similar to that described for human β_2m (Karlsson, 1974; Isenman et al., 1975; Lancet et al., 1979; Trägardh et al., 1979). The murine β_2m primary structure is 69% homologous to the human at the protein level (Kimball & Coligan, 1983).

Theoretical predictions of secondary structure based on amino acid sequences, although limited in accuracy (Kabsch & Sander, 1983), are often of value for estimating the secondary structural properties of proteins for which crystallographic, three-dimensional analyses have not been done. The theoretical (predicted) values for the H-2K^b heavy-chain papain fragment, based on a Chou–Fasman analysis, were 36% α-helix, 29% β-sheet, and 35% random coil. These amounts are consistent with the values determined from the CD spectrum (Table II).

The schematic diagram (Figure 3) of the predicted secondary structure for the complete heavy chain is of considerable interest as it suggests that the large amounts of α-helix and β-sheet structure are distributed throughout the molecule with an α-helical transmembrane region similar to what has been indicated for other membrane-associated molecules (Robb et al., 1978; Schulte & Marchesi, 1979; Rogers et al., 1980). It also suggests that the heavy chain is organized into regions containing large amounts of α-helix and β-sheet structure separated by β-turns, which is consistent with previous studies of the protein and gene organization, suggesting discrete domains (Nathenson et al., 1981; Steinmetz & Hood, 1983; Weiss et al., 1983). Each domain is encoded by a separate exon at the DNA level (Figure 3). Although they were initially defined structurally, the domains are also functionally separable. β_2m binds to the α3 domain and alloreactive specificities seem to be located primarily in α1 and α2 (Nairn et al., 1980; Hämmerling et al., 1982; Sherman, 1982; Clark & Forman, 1983; Ozato et al., 1983; Reiss et al., 1983; Yokoyama & Nathenson, 1983; Allen et al., 1984).

At present, it is not possible to further speculate as to the role in terms of functional properties of the specific regions of α-helix and β-sheet structure within the domains. Of interest, however, are the findings for the α3 domain. A possible evolutionary relationship between histocompatibility antigens and immunoglobulins has been suggested by the amount of sequence homology (approximately 20%) between β_2m, the α3 domain of H-2 and HLA molecules, and the C_γ3 domain of immunoglobulins (Bodmer, 1972; Gally & Edelman, 1972; Karlsson, 1974; Peterson et al., 1974). The CD spectra for H-2K^b, HLA antigens, and immunoglobulins also exhibit some similarity, with a minimum near 219 nm, although the H-2K^b spectrum also has a minimum at 214 nm (Figure 3) (Dorrington & Tanford, 1970; Bjork et al., 1971; Isenman et al., 1975; Tärgardh et al., 1981). A Chou–Fasman analysis comparing the α3 domain of H-2K^b and HLA-B7 with C_γ3 suggested a high degree of secondary structural similarity in this region. All three protein segments contain large amounts of β-sheet structure, although both H-2K^b and HLA-B7 contain more α-helix than the immunoglobulin domain. These structural differences may be associated with the distinct functional difference between the α3 domain of histocompatibility antigen and the C_γ3 domain of immunoglobulins.

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Monoclonal Antibodies against the Membrane-Bound, Flavin-Linked D-Lactate Dehydrogenase of *Escherichia coli*: Preparation, Characterization, and Use in Immunoaffinity Chromatography

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ABSTRACT: Three mouse hybridoma cell lines are described that produce monoclonal antibodies directed against the membrane-bound, flavin adenine dinucleotide linked D-lactate dehydrogenase of *Escherichia coli*. In contrast to polyclonal antibodies produced in rabbits, none of the monoclonal antibodies inhibits enzyme activity. Immunoblots of D-lactate dehydrogenase proteolytic fragments indicate that each antibody is directed against a different region of the molecule. One monoclonal antibody, 1B2a, reacts with native, undigested D-lactate dehydrogenase only and is used to purify the enzyme in a single step. The protocol involves chromatography of a Triton X-100 extract of membrane vesicles containing D-lactate dehydrogenase on a column made with the monoclonal antibody coupled to a solid support. After the column is washed free of unadsorbed protein, elution at high pH in the presence of guanidine hydrochloride yields a fraction containing highly purified, catalytically active D-lactate dehydrogenase.

Active transport of many different solutes by right-side-out plasma membrane vesicles from various bacteria is driven by a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline) (Kaback, 1974, 1976, 1983, 1985; Konings & Boonstra, 1977) generated by means of substrate oxidation via a membrane-bound respiratory chain. Although vesicles have the capacity to oxidize a variety of substrates, generation of $\Delta\bar{\mu}_{H^+}$ seems to be relatively specific for certain electron donors. Thus, D-lactate is the most effective physiological electron donor for generating $\Delta\bar{\mu}_{H^+}$ in *Escherichia coli* ML 308-225 membrane vesicles, even though its rate of oxidation is slower than that of other electron donors such as NADH or succinate (Barnes & Kaback, 1971; Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975).

E. coli membrane vesicles containing D-lactate dehydrogenase (D-LDH) catalyze the stoichiometric conversion of D-lactate to pyruvate (Kaback & Milner, 1970; Barnes & Kaback, 1970), and electrons derived from this reaction are transferred to oxygen through membrane-bound respiratory intermediates. Concomitant with electron flow, a transmembrane $\Delta\bar{\mu}_{H^+}$ is generated by a mechanism(s) that is (are) not completely understood (Kaback, 1985). In any event, D-LDH is readily solubilized from the membrane with chaotropic agents or nonionic detergents and readheres to the membrane in a functional manner upon dilution of the solubilizing agent (Reeves et al., 1973; Short et al., 1974, 1975b; Haldar et al., 1982). The enzyme has been purified to homogeneity in at least three different laboratories (Kohn & Kaback, 1973; Kaczorowski et al., 1978; Futai, 1973; Pratt et al., 1979). In each instance, it exhibits an apparent molecular weight of about 65 kdaltons on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and is composed of a single

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; D-LDH, D-lactate dehydrogenase; DCIP, 2,6-dichlorophenolindophenol; Mab, monoclonal antibody; PBS, phosphate-buffered saline; Pen–Strep, penicillin–streptomycin; HAT, hypoxanthine/aminopterin/thymidine; pristane, 2,6,10,14-tetramethylpentadecane; SP-RIA, solid-phase radioimmunoassay; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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