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Inhibition of the Gelation of Extracellular and Intracellular Hemoglobin S by Selective Acetylation with Methyl Acetyl Phosphate†

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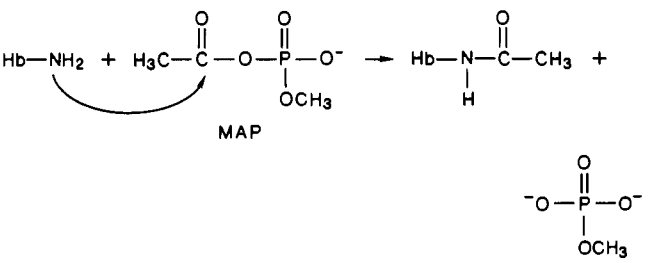
ABSTRACT: Methyl acetyl phosphate binds to the 2,3-diphosphoglycerate (2,3-DPG) binding site of hemoglobin and selectively acetylates three amino groups at or near that site. The subsequent binding of 2,3-DPG is thus impeded. When intact sickle cells are exposed to methyl acetyl phosphate, their abnormally high density under anaerobic conditions is reduced to the density range of oxygenated, nonsickling erythrocytes. This change is probably due to a combination of direct and indirect effects induced by the specific acetylation. The direct effect is on the solubility of deoxyhemoglobin S, which is increased from 17 g/dL for unmodified hemoglobin S to 22 g/dL for acetylated hemoglobin S at pH 6.8. Acetylated hemoglobin S does not gel at pH 7.4, up to a concentration of 32 g/dL. The indirect effect could be due to the decreased binding of 2,3-DPG to deoxyhemoglobin S within the sickle erythrocyte, thus hindering the conversion of oxyhemoglobin S to the gelling form, deoxyhemoglobin S.

Methyl acetyl phosphate (MAP),[1] a monoanionic acetylating agent, was designed (Kluger & Tsui, 1980) for the active site of those enzymes that bind small anions and have a susceptible nucleophile nearby. We have previously shown that MAP reacts with hemoglobin A (HbA) in a very specific fashion (Ueno et al., 1986). Thus, for oxy-HbA there were only three amino groups per $\alpha\beta$ dimer that were acetylated, and these were Val-1, Lys-82, and Lys-144 of the β-chain.



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These amino acid residues are at or near the binding site for 2,3-diphosphoglycerate (2,3-DPG), and they include all of the amino groups in this cleft.

The specificity of the reagent was further demonstrated by the fact that none of the amino groups of the α-chain were acetylated in oxy-Hb. These findings provided evidence for the suggestion that MAP is a site-directed acetylating agent due to the prior binding of its phosphate moiety in the DPG cavity.

Since MAP reacts with Val-1(β) and carbamylation at this site by sodium cyanate results in an increased gelling concentration of deoxy-HbS (Nigen et al., 1974), we have tested the gelation behavior of acetylated HbS. It also seemed desirable to test the effect of MAP on intact sickle cells in order to determine the permeability of the compound and also whether acetylation at the DPG binding site would preclude binding of this allosteric effector and thus shift the oxygen equilibrium curve to the oxy, nonsickling conformation. Such studies are described in this paper.

Materials and Methods

Synthesis of Methyl Acetyl Phosphate. This compound was prepared by a minor modification of the method of Kluger and Tsui (1980). The desired compound, which was recrystallized from methanol–ether, had the expected NMR spectrum and elemental analysis. The latter procedure was skillfully performed by S. T. Bella of this institution.

Preparation and Treatment of Sickle Cells with MAP. Whole blood, obtained by venipuncture from individuals homozygous for sickle cell anemia, was centrifuged at 2000*g* for 10 min. The plasma was separated and retained for reconstitution with the sickle erythrocytes for subsequent cell density experiments. For the other studies, the red cells were washed twice with 0.1 M NaCl in 50 mM Hepes, pH 7.5, and the washed cells were resuspended to their original volume in the same buffer.

After treatment of washed erythrocytes (1 mM Hb) with MAP (50 mM) for 1–3 h at 37 ° C, the red cells were isolated by centrifugation and washed as described above. The Hb was then extracted by lysis of the cells in 2 volumes of glass-distilled water. After centrifugation of the lysate at 7800*g* for 20 min for removal of red cell debris, the isolated Hb was either extensively dialyzed against 0.1 M NaCl or further purified as described below.

HPLC Chromatography of Acetylated Hemoglobin S. The dialyzed red cell lysate obtained from MAP-treated sickle erythrocytes as described above was applied directly to a cation-exchange resin in order to approximate the extent of reaction. Since acetylation of amino groups reduces the net positive charge of the protein, it seemed reasonable to assume that chromatography on a cation-exchange resin would be a good measure of the amount of reaction of HbS with MAP within intact sickle cells. Therefore, aliquots of the Hb isolated from MAP-treated cells were injected onto a MA7C HPLC column (4.6 × 30 mm, Bio-Rad, Richmond, CA) on a Spectra Physics 8700 HPLC system. The column was equilibrated with 30 mM Bis-Tris–1.5 mM potassium acetate, pH 6.40, and developed with 30 mM Bis-Tris, 1.5 mM potassium acetate, and a gradient of 0–100% 150 mM sodium acetate, pH 6.40, at a flow rate of 1.5 mL/min. The elution of Hb was monitored by its absorption at 415 nm.

Purification of Hemoglobin S. For experiments in which the effect of DPG on acetylated Hb was determined, the Hb was first purified by ion-exchange chromatography for removal of minor Hb components by the procedures described previously (Acharya & Manning, 1980) before treatment with MAP. The acetylated HbS was isolated on CM-52 (Ueno et al., 1986). The purified Hb fractions were pooled, and the concentration of oxy-Hb was determined by its absorption at 540 nm ($\epsilon = 57.08 \times 10^3$ M^{-1} cm^{-1}). The absence of met-Hb was established by the lack of any absorbance at 630 nm.

Determination of the Oxygen Equilibrium Curves of Acetylated Hemoglobin. The dialyzed lysate containing acetylated HbS was again dialyzed against 50 mM Bis-Tris–acetate, pH 7.4, or 0.1 M potassium phosphate, pH 6.8, and concentrated to about 0.5 mM (in Hb tetramer) on an Amicon ultrafiltration apparatus. The oxygen equilibrium curves were determined in the same buffer at 37 °C on an Aminco Hem-O-Scan instrument. Experiments in the presence of 2,3-DPG were performed on HbS samples that had been previously mixed with the organic phosphate in increasing ratios of DPG to Hb. Prior to use, the 2,3-DPG was subjected to chromatography as described previously (Nigen & Manning, 1975). The P_{50} values were determined directly from the graphs of the Hem-O-Scan.

Determination of the Gelling Concentration (C^*). The onset of gelation (C^*) was determined as described by Benesch et al. (1978). The P_{50} values of Hb samples at concentrations varying from 5 to 40 g/dL were determined in 0.1 M potassium phosphate, pH 6.8, or in 0.05 M Bis-Tris–acetate, pH 7.4, at 37 °C on the Hem-O-Scan instrument. The onset of gelation is measured by a sudden decrease in oxygen affinity such that a biphasic line is obtained. The point of intersection of these lines represents the C^*.

Determination of Cell Densities of Untreated and Methyl Acetyl Phosphate Treated Sickle Cells. Cell density fractionation was performed according to the procedure of Danon and Marikowski (1964). Methyl phthalate ($\rho = 1.189$) and di-*n*-butyl phthalate ($\rho = 1.0416$), obtained from Fisher Scientific, were mixed in varying ratios to prepare 20 solutions ranging in density from 1.062 to 1.138 with increments of 0.004 between each solution.

To determine cell density changes, 20 heparinized capillary hematocrit tubes were filled with 5 μL of each of the phthalate ester mixtures. Then, 50 μL of untreated or treated cells (50 mM MAP, 3 h, 37 °C, and reconstitution in citrate–phosphate–dextrose plasma) was drawn into each capillary tube. One end was sealed with Seal-Ease (Clay-Adams), and the tubes were then centrifuged for 15 min at 12000*g* in a microhematocrit centrifuge so that cells denser than the specific gravity of the ester equilibrated at a point lower than that of less dense cells. The densities were plotted on the X axis, and the percent red cells below the ester mixture was plotted on the Y axis to determine the relative densities of treated vs. untreated cells. The percentage of cells below the ester of median density (1.10) and the specific gravity at which 50% of the cells below the ester mixture (D_{50}) were extrapolated from the graphs.

Results

Incorporation of Methyl Acetyl Phosphate into Sickle Cells. Washed intact sickle cells were treated with 50 mM MAP under aerobic conditions for 1.5 h at 37 °C. There was no evidence of cell lysis as determined by the absorbance at 540 nm of the supernatant after centrifugation of the treated cells.

[1] Abbreviations: MAP, methyl acetyl phosphate; Hb, hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; P_{50}, pressure at which hemoglobin is 50% saturated with O_2; D_{50}, specific gravity of phthalate esters at which 50% of sickle cells are more dense; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane.

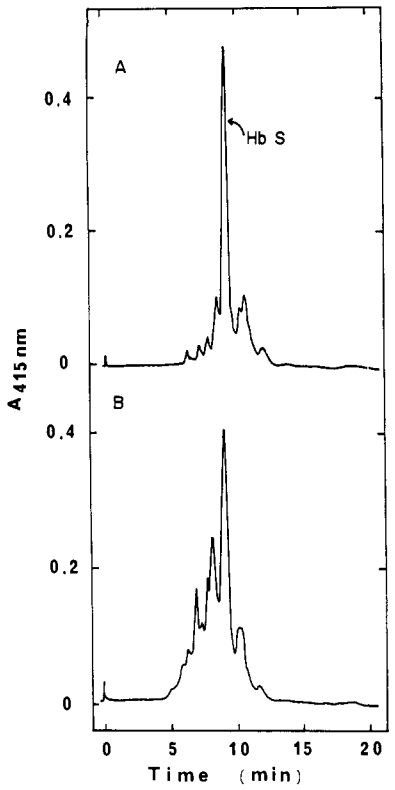

FIGURE 1: HPLC separation of lysates of erythrocytes from a patient with sickle cell anemia: (A) −MAP; (B) +MAP. The treatment of the cells and chromatographic conditions are described under Materials and Methods.

To approximate the extent of reaction of the intracellular Hb, HPLC analysis as described above was performed (Figure 1). As shown in panel A, the extract from untreated cells shows a predominant peak of HbS and several smaller peaks, which most likely represent the various glycosylated and minor Hb derivatives. The HbS extracted from a similar aliquot of sickle cells that had been treated with MAP is shown in panel B. The predominant peak of HbS is diminished by 40%, but the amounts of the more acidic components are increased significantly. These results are similar to previous findings that we have obtained with isolated HbA in which the acidic components were shown to be acetylated HbA (Ueno et al., 1986). Thus, these components are most likely those HbS tetramers that have been acetylated by MAP. In the previous study, these components were purified by ion-exchange chromatography, and the sites of acetylation were assigned by differential peptide mapping on HPLC (Ueno et al., 1986). From the present results, we can conclude that MAP traverses the red cell membrane and reacts with HbS within the cell.

Effects of Methyl Acetyl Phosphate on the Oxygen Affinity of Hemoglobin S in the Presence or Absence of 2,3-DPG. These experiments were performed on the Hb derivative that had the highest degree of acetylation as determined by its chromatographic mobility on CM-52 (Ueno et al., 1986). In the *absence* of any added 2,3-DPG, the HbS that had been treated with MAP had a lower intrinsic oxygen affinity than did unmodified Hb (Figure 2). This finding is consistent with the generalization that any reduction in the net positive charge around the 2,3-DPG binding site leads to a lower oxygen affinity, as found with Hb Raleigh, which is acetylated at the NH_2 terminus of the β-chain (Bonaventura & Bonaventura, 1978), and in Hb carbamylated at the same position (Nigen et al., 1974).

For the Hb treated with MAP and purified on CM-52 as described above, the effect of added 2,3-DPG on the oxygen affinity of the acetylated Hb is marginal even at high molar ratios of DPG to Hb (Figure 2). This result indicates that acetylation at the 2,3-DPG cleft inhibits subsequent binding of the organic phosphate. For unmodified Hb, addition of 2,3-DPG causes a significant decrease in the oxygen affinity. The maximum effect is reached at about a molar ratio of DPG/Hb of 1:1 and changes very little upon further addition of 2,3-DPG, as observed previously (Benesch & Benesch, 1967).

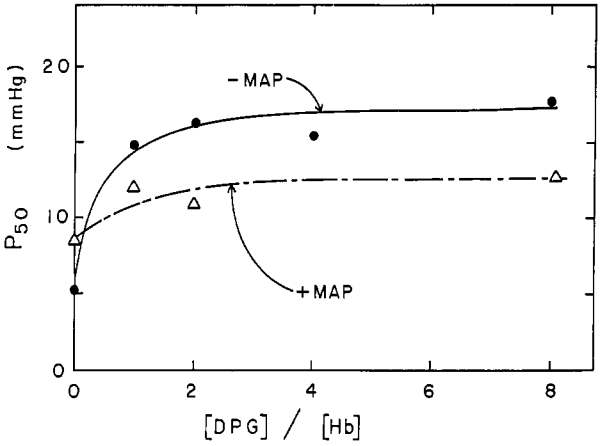

FIGURE 2: Effect of 2,3-DPG on the P_{50} of unmodified and of acetylated HbS. Oxy-HbS (0.1 mM) was purified on DE-52 as described in the text and then was treated with MAP (1 mM) in 50 mM Hepes, pH 7.5, at 25 °C. After extensive dialysis against 10 mM potassium phosphate, pH 5.85, modified HbS was subjected to further purification on a CM-cellulose column as described in the text. The oxygen affinity was measured in the presence or absence of DPG as described in the text.

Effect of Methyl Acetyl Phosphate on the Gelation of Deoxyhemoglobin S. The gelation of unmodified deoxy-HbS, as measured by the Benesch procedure (Benesch et al., 1978), occurs at a concentration of about 17–18 g/dL (Figure 3). However, at pH 6.8 the acetylated Hb gels at a higher concentration, about 22 g/dL (panel B). At pH 7.4 there is no detectable gelation of acetylated HbS at least up to a concentration of 32 g/dL (panel A). These results indicate that acetylation of the amino groups in the DPG pocket of HbS significantly reduces the propensity of deoxy-HbS to gel.

Effect of Methyl Acetyl Phosphate on the Density of Intact Sickle Cells. The density profile of cells treated with 50 mM MAP for 3 h was compared with that of cells treated with buffer under aerobic or anaerobic conditions (Figure 4). Under conditions of complete oxygenation, cells untreated or treated with MAP showed nearly identical density profiles (panel A). This result indicates that treatment with MAP does not lead to an increase in intracellular water content. Under anaerobic conditions, untreated cells showed a marked shift to the right, reflecting an increase in cell densities due to gelation of intracellular deoxy-HbS (panel B). In contrast, deoxygenated cells that had been treated with MAP prior to cell density fractionation had a profile that indicated a significantly less dense population of cells. Furthermore, the density profile of treated cells under anaerobic conditions is identical with that of the oxygenated cells. Extrapolation of the percentage of cells below the ester of median specific gravity, 1.10, indicated that 76% of both oxygenated MAP-treated cells and untreated cells (panel A), 75% of deoxygenated MAP-treated cells, and 88% of deoxygenated untreated cells were more dense. Determination of D_{50} indicated a similar trend. Thus, for oxygenated cells (either treated or untreated) and for deoxygenated, treated cells, the D_{50} was 1.105 whereas for deoxygenated untreated cells the D_{50} was

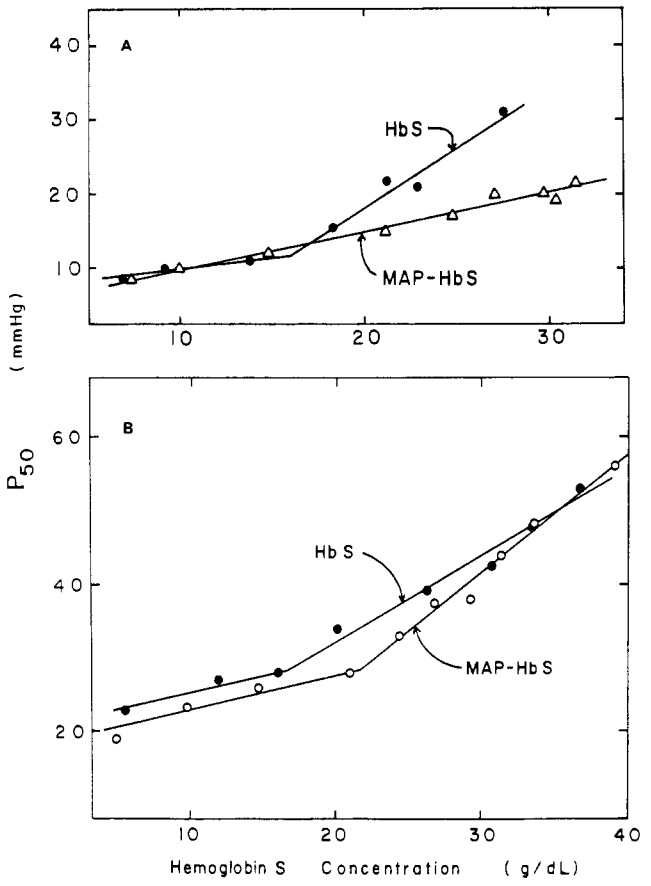

FIGURE 3: Relation between P_{50} and concentration of HbS isolated from untreated or MAP-treated erythrocytes of patients with sickle cell anemia. Erythrocytes were treated with MAP, and the red cell lysate was obtained. The lysate was dialyzed against 0.1 M NaCl and then against the appropriate buffer. After concentration to 34–40 g/dL, the P_{50} was measured at 37 °C on an Aminco Hem-O-Scan. (A) The P_{50} of different concentrations of untreated and acetylated HbS in 50 mM Bis-Tris–acetate buffer, pH 7.4, at 37 °C. (B) The P_{50} of different concentrations of untreated and acetylated HbS in 0.1 M potassium phosphate buffer, pH 6.8, at 37 °C.

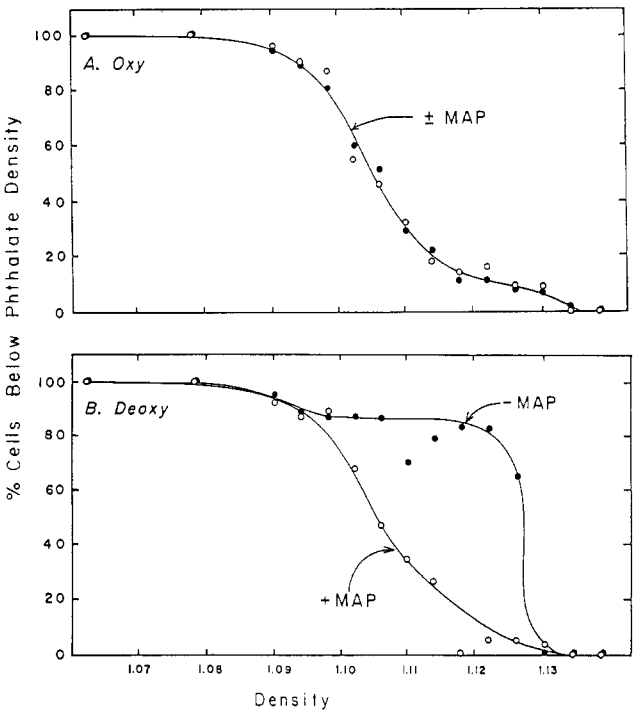

FIGURE 4: Effect of MAP on the cell density of sickle cells in the presence or absence of oxygen. Anaerobic conditions were achieved in a glovebag with an atmosphere of N_2. The density gradient with mixtures of phthalate esters was produced with two esters of different specific gravity as described in the text. (A) Cell density was measured under aerobic conditions for MAP-treated (○) or for untreated (●) sickle cells. (B) Cell density was measured under anaerobic conditions for MAP-treated (○) or for untreated (●) cells.

1.126.

Discussion

MAP was originally designed for studies on the anion binding sites of proteins (Kluger & Tsui, 1980, 1986). The strategy was that the monoanionic phosphate group would bind to the cation binding site of a dehydrogenase and subsequently acetylate a nearby nucleophile. Kluger and Tsui showed, with several different dehydrogenases, that MAP inactivated these enzymes fairly rapidly with a half-life in the range of 20–30 min. This compound is quite stable at neutral pH, and unlike its parent compound acetyl phosphate (which is not an acetylating agent but rather is a phosphorylating agent), MAP is an acetylating agent but not a phosphorylating reagent.

In this study we have demonstrated that MAP readily traverses the membrane of the sickle erythrocyte and reacts with intramolecular HbS to inhibit gelation of the protein. The presence of acetylated HbS within the erythrocyte interferes with the aggregation process such that the density of the sickle cell population no longer displays the shift toward a higher profile characteristic of the sickling process. The fact that the profile of the treated cells is nearly the same as that of oxygenated cells indicates that the inhibition of gelation within the cell must be quite efficient. It is probable that this decrease in cell density comes about not only *directly* because of an effect on the gelation process as described above but also *indirectly* because of reduced binding of 2,3-DPG to the protein as also shown above. The latter mechanisms would disfavor the formation of the T state of Hb and subsequent aggregation within the cell.

The profile of reaction of MAP with Hb indicates that it has considerably more specificity than other acetylating agents such as aspirin previously used with Hb. Its degree of specificity is reminescent of that found for the bifunctional aspirin derivatives that have been designed to react with the two Lys-82 residues of the DPG binding site in oxy-Hb (Walder et al., 1977). In the latter case, the product is a disubstituted cross-linked Hb. With MAP, there is no such cross-linking, and monoacetylation of the protein is achieved.

The fact that so few of the amino groups of the β-chains (Val-1, Lys-82, and Lys-144) and that none of the amino groups of the α-chain are acetylated by MAP under aerobic conditions is also indicative of the selectivity of this reagent. It is especially noteworthy that even Val-1(α) is not acetylated under such conditions. These results indicate that MAP cannot bind near this latter site in oxy-Hb. Even though 2,3-DPG binds less tightly to the oxy conformation of Hb than it does to the deoxy conformation, the binding of MAP in this cleft is apparently of sufficient magnitude to permit subsequent acetylation. Therefore, it appears likely that binding must precede acetylation. The propensity of the DPG cleft to bind inorganic anions is well established (Antonini et al., 1972; Arnone & Williams, 1977; Bare et al., 1972; Chiancone et al., 1972; Nigen et al., 1980).

The direct effect of acetylation on the gelation of HbS could be due to blockage of the N-terminal valine residue of the β-chain of HbS analogous to the effect of carbamylation at this site (Nigen et al., 1974). It is conceivable that acetylation of Lys-82(β) or Lys-144(β) also has a direct effect on the solubility of deoxy-HbS, but this possibility must await further

study with the specific derivatives.

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Characterization of Disulfide Bonds in Recombinant Proteins: Reduced Human Interleukin 2 in Inclusion Bodies and Its Oxidative Refolding

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ABSTRACT: Cloned cDNA of human interleukin 2 (IL-2) was expressed in *Escherichia coli* cells in which IL-2 formed insoluble inclusion bodies. Human IL-2 has three Cys residues, namely, Cys-58, Cys-105, and Cys-125, and native IL-2 has an intramolecular disulfide bond between Cys-58 and Cys-105. Since the formation of inclusion bodies was thought to be due to disorder in the oxidation state of these Cys residues, all intramolecular disulfide bond isomers of IL-2 were prepared by denaturation of native IL-2 to characterize the state of a disulfide bond in IL-2 in the inclusion bodies. These isomers can be separated from native IL-2, reduced IL-2, and IL-2's with intermolecular disulfide bonds by means of reversed-phase high-performance liquid chromatography. Human IL-2 produced in inclusion bodies in *E. coli* carrying a recombinant DNA was analyzed by HPLC and was proved to be a fully reduced form with no intra- and intermolecular disulfide bonds. Refolding of reduced IL-2 in the presence of reduced and oxidized glutathione and a low concentration of guanidine hydrochloride resulted in the formation of the biologically active IL-2 quantitatively. Further purification provided a practically pure IL-2 preparation without contamination of any disulfide bond isomers.

Interleukin 2 (IL-2),[1] also referred to as T-cell growth factor, is a lymphokine produced by activated T-cells (Morgan et al., 1976; Gillis et al., 1979). The important biological activities of this protein are reported to be promotion of long-term in vitro proliferation of antigen-specific effector T-lymphocytes and induction of cytotoxic T-lymphocyte reactivity (Gillis & Smith, 1977; Watson, 1979; Gillis et al., 1980; Watson et al., 1979). Interest in IL-2 has increased recently owing to reports of a possible role for this lymphokine in the treatment of solid tumors (Rosenberg et al., 1985). Recently, mRNAs for human IL-2 from various sources, such as a leukemic T-cell line (Taniguchi et al., 1983), spenocytes (Devos et al., 1983), and tonsillar mononuclear cells (Maeda et al., 1983), were isolated and their cDNAs were cloned and sequenced. Cloned cDNA, when inserted in *Escherichia coli*, was expressed at high concentration, and the expressed protein was purified by

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[1] Abbreviations: IL-2, interleukin 2; *E. coli*, *Escherichia coli*; HPLC, high-performance liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; DTT, 1,4-dithiothreitol; Cm, carboxymethylated; CTLL, cytotoxic T lymphocyte line; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; re-IL-2, reduced IL-2; IL-2(105–125), IL-2 with a disulfide bond between Cys-105 and Cys-125; IL-2(58–125), IL-2 with a disulfide bond between Cys-58 and Cys-125; IL-2(58–105), IL-2 with a disulfide bond between Cys-58 and Cys-105; BPTI, bovine pancreatic trypsin inhibitor.

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