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ERYTHROPOIETIN FORMS BIOLOGICALLY INACTIVE COMPLEXES IN SOLUTION

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SUMMARY: Erythropoietin, isolated from human urine, exhibits a time-dependent increase in biological activity in solution. This increase is not due to the action of proteolytic cleavage of an inactive precursor molecule. Gel filtration chromatography demonstrates that erythropoietin exists in at least two forms: (1) biologically active, (2) biologically inactive and complexed with one or more other proteins. Dissociation of erythropoietin from this complex results in the observed time-dependent increase in activity. This behavior bears directly on studies of the hormone's physicochemical and biological properties and may reflect a mechanism for the control of hormonal activity.

INTRODUCTION: Erythropoietin is a glycoprotein hormone which is necessary for the proliferation and differentiation of avian and mammalian red blood cell progenitors (1). The mechanism by which the hormone acts is unknown. The primary site of synthesis of erythropoietin appears to be the kidney (2,3), and it is probably released in an active form. However, an alternative hypothesis, namely that the kidney produces a proteolytic enzyme which cleaves a plasma protein, thus liberating biologically active erythropoietin, has not been conclusively ruled out (4). Clearly, the elucidation of the biochemistry and cellular physiology of the hormone ultimately requires that its physicochemical characteristics and behavior in solution be known with certainty. The results of the present study indicate that erythropoietin can exist in an inactive complex and that the active hormone dissociates from the complex relatively slowly, thus appearing as a time-dependent increase in specific activity.

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MATERIALS AND METHODS: Erythropoietin was prepared from the urine of anemic human donors collected in the presence of 0.01% NaN_3 and stored at -20°C. The urinary protein was concentrated by ultrafiltration on a hollow fiber device (Amicon Model DC-2) with a nominal MW cutoff of 10,000 (H1P-10 cartridge). The concentrate was desalted by gel filtration through Bio-Gel P-4 equilibrated with distilled H_2O. The protein peak (void volume) contained all of the erythropoietin activity. Erythropoietin activity was measured by a modification of the *in vitro* method of Stephenson and Axelrad (5) employing mouse bone marrow instead of fetal liver. Erythropoietin-dependent hemoglobin synthesis was assessed as follows. Portions of bone marrow cells (5-7 x 10^5 nucleated cells each) obtained from the femurs of 6-12 week-old female CD-1 mice were incubated in Falcon Multiwells for 20 hr at 37°C, 95% air/5% CO_2 in 0.5 ml of growth medium containing 90% NCTC-109 (GIBCO), 10% fetal calf serum (Microbiological Assoc.), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin with or without erythropoietin. Three microcuries of ^{59}Fe (New England Nuclear) in 10 µl of mouse serum (Miles Biochemicals) were then added to each well, and the cultures were incubated an additional 5 hr. The cells were washed free of excess ^{59}Fe with 1 ml of Dulbecco's phosphate buffered saline and lysed with 1 ml of distilled H_2O. The liberated ^{59}Fe-hemoglobin was converted to ^{59}Fe-cyanmetheme with 2 ml of Drabkin's solution (Fisher) and 2 ml of 0.1 N HCl. The solution was extracted with 5 ml of cyclohexanone and the ^{59}Fe-cyanmetheme in the organic phase was measured by gamma scintillation spectrometry (Tracor, Model 1185). NIH erythropoietin lot M-7-TaLSL (16 U/mg) (generous gift of Dr. David G. Nathan) was used as a standard. Protein concentrations were measured at 278nm with a Gilford Model 2400 spectrophotometer. For this study an extinction coefficient $E_{278}^{0.1\%} = 1.0$ determined by protein dry weight (6) on total urinary proteins has been employed.

RESULTS AND DISCUSSION: The specific activity of human urinary erythropoietin increases as a function of time in solution (Figure 1). Two milligrams of lyophilized urinary protein were dissolved in 1 ml of 50 mM Tris-HCl, pH 7.4,

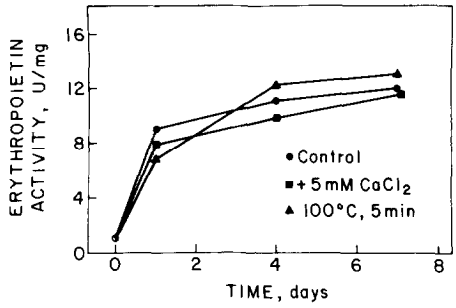

Figure 1: Time-dependent increase in erythropoietin activity. Conditions: A (●), 50 mM Tris-HCl, pH 7.4, 4°C; B (■), as A plus 5 mM $CaCl_2$; C (▲), 100°C for 5 min, then as A.

4°C and the solution was assayed for erythropoietin activity at 0, 1, 4 and 7 days. The specific activity increased from 1 U/mg to a maximum of 12 U/mg in the 7 day interval and was stable for two weeks thereafter.

The addition of 5 mM Ca^{2+}, necessary for the activity of several proteases, did not increase the rate of appearance of activity. Similarly, initial heating of a replicate sample for 5 min at 100°C to denature any proteases did not significantly alter the kinetics of the activity increase. Lastly, no proteolytic activity was demonstrated using p-toluenesulfonyl-L-arginine methyl ester, N-benzoyl-L-arginine ethyl ester, leucine amide or hippuryl-L-phenylalanine as substrates. These data, taken together, strongly suggest that the time-dependent increase in erythropoietin activity was not due to proteolytic cleavage of a precursor protein with liberation of the active hormone.

Gel filtration chromatography demonstrates that erythropoietin exists at least in part complexed to one or more other proteins and that it is the dissociation of the hormone from this complex which explains the time-dependent activity increase. Samples of human urinary erythropoietin (5.4 mg in 1 ml) were incubated in 50 mM Tris-HCl, pH 7.4, 4°C for 1 or 7 days, conditions shown above to permit either partial or complete appearance of activity, respectively, and gel filtered through Bio-Gel P-200 (Figure 2A, B, C). The fractions were then either assayed immediately for erythropoietin activity (Fig. 2A and C) or kept at 4°C for an additional 4 days to allow completion of any

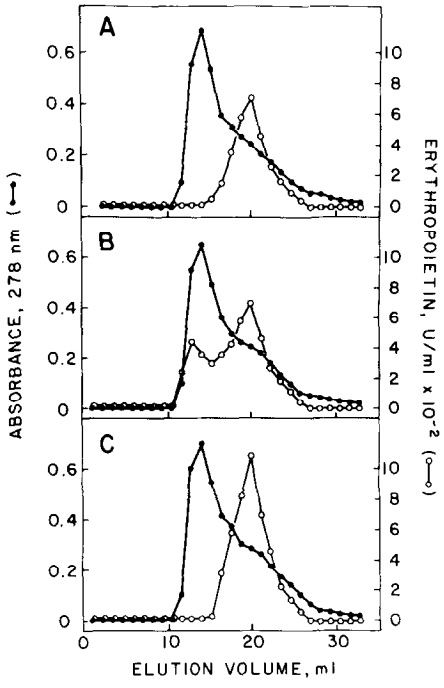

Figure 2: Gel filtration chromatography of human urinary erythropoietin. Panel A, protein solution incubated 1 day at 4°C, chromatographed and assayed; Panel B, protein solution incubated 1 day at 4°C, chromatographed, incubated 4 days at 4°C and assayed; Panel C, protein solution incubated 7 days, chromatographed and assayed. Sample, 5.4 mg protein in 1 ml; buffer, 50 mM Tris-HCl, pH 7.4; column Bio-Gel P-200, 100-200 mesh, 43 ml volume, fractions = 1.1 ml. See text.

further dissociation of active hormone with concomitant appearance of activity and then assayed (Fig. 2B). After only one day in solution prior to chromatography and assayed immediately, erythropoietin activity was found in a single symmetrical peak with an elution volume Ve = 20 ml (Fig. 2A). However, when the chromatography fractions from a replicate sample were incubated at 4°C for 4 days prior to assay (Fig. 2B), a new activity peak was found at Ve = 13 ml in addition to the peak at Ve = 20 ml. Importantly, the total units under the Ve = 20 ml peaks in both experiments are very similar, 2570 and 3100 U, respectively. Thus, the additional 1330 U found under the new 13 ml peak appeared during the 4 days of incubation prior to assay, suggesting dissociation of active hormone from an inactive complex of higher molecular weight. After concentration by ultrafiltration and rechromatography, the new Ve = 13 ml peak eluted as a single peak centered at 20 ml, confirming this conclusion. This

is further supported by the results obtained when an identical sample was incubated for 7 days prior to chromatography, conditions which result in complete dissociation of the complex (Fig. 2C). A single peak of activity eluted from the column with a Ve = 20 ml. However, the total activity under this peak was increased to 4380 U, in striking agreement with the sum of the 13 ml and 20 ml peaks of the previous experiment, 4430 U. Thus, erythropoietin, which eluted at 20 ml under the chromatographic conditions employed, can exist in a biologically inactive complex of higher molecular weight eluting at 13 ml. The active hormone dissociates slowly from the complex under these conditions and can again be shown to elute at 20 ml.

The nature of this association-dissociation process is at present not defined. It may reflect a mechanism for the control of erythropoietin activity *in vivo* characterized by an equilibrium between free and bound erythropoietin corresponding to active and inactive hormone. If this is the case, the association necessarily must possess great specificity, similar to that of an antigen-antibody complex. Moreover, some mechanism for control of the equilibrium is required. Alternatively, the association may reflect aggregation of the hormone with itself. Indeed, this phenomenon has been shown with the hormone glucagon (7) which appears to proceed from monomer to dimer to hexamer depending upon experimental conditions (8). Other studies, however, suggest that a trimer is the preferred state of aggregation (9).

Finally, this phenomenon may be the result of the general propensity of glycoproteins to associate, presumably through their sugar moieties. In this regard, it must be pointed out that it is not yet known whether these observations on the behavior of human urinary erythropoietin are applicable to the hormone as it exists in the plasma. Further work may reveal the importance of the erythropoietin protein complex to the hormone's interaction with the cell surface.

Of some interest is the discrepancy between the data reported here and those previously reported by Chiba *et al* (10). These investigators observed

an inactivation of urinary erythropoietin stored at -20°C for several weeks. Furthermore, the authors reported protease activity in their preparation, using an insoluble collagen-dye complex (Azocoll) as substrate. Treatment of the erythropoietin with phenol and p-aminosalicylic acid reduced the apparent protease activity by 74% and completely prevented inactivation at -20°C. Substantial loss of initial hormone activity has since been reported using this method. No such inactivation of erythropoietin was observed in the course of the present study or subsequently. It is possible that the discrepancy is due to differences in the initial isolation procedure.

The present data demonstrate that erythropoietin can exist as an inactive protein-protein complex which slowly dissociates in solution under the conditions employed. It is thus important to consider such protein-protein interactions and the existence of erythropoietin in one or more states of association when undertaking studies of the hormone's physicochemical or biological properties.

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