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DENATURATION AND RENATURATION OF HUMAN ERYTHROPOIETIN

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SUMMARY: Human erythropoietin can be denatured with 6M urea or with 6M urea/1% sodium dodecyl sulfate and renatured with restoration of biologic activity. Activity cannot be restored if the denatured hormone is exposed to 10mM 2-mercaptoethanol strongly suggesting the existence of one or more "buried" disulfide bonds critical for biologic activity. Polyacrylamide gel electrophoresis under denaturing conditions resulted in an apparent molecular weight of 25,000, significantly lower then recent estimates.

INTRODUCTION: An understanding of the molecular basis of erythropoiesis ultimately requires the elucidation of the mechanism of action of erythropoietin, a polypeptide hormone which is necessary for the maturation of erythroid progenitors. Despite years of study, relatively little is known with certainty about the physicochemical properties of the hormone (1-4).

An examination of the denaturation and renaturation of a protein can result in important structural information which cannot be derived from studies limited to the protein's native conformation. For example, amino acid residues critical to a protein's function may react with group-specific reagents and, hence, be detected only after the protein conformation is so altered. Similarly, reliable estimates of the molecular weight of many proteins have been obtained only after denaturation (5-11). Such approaches may be particularly relevant to erythropoietin since recent work indicates that it can exist in solution complexed to one or more other proteins (12). Those reagents used to denature the protein also serve to eliminate those weak interactions upon which such

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protein-protein complexes are based. The present study reports the results of denaturation and renaturation of human erythropoietin by urea and sodium dodecyl sulfate.

MATERIALS AND METHODS

Erythropoietin: The hormone was prepared from the urine of anemic human donors (12). Desalted protein concentrates were either lyophilized and stored at -80°C or concentrated by ultrafiltration and subjected to one or more further purification steps (4). Erythropoietin activity was determined by an *in vitro* mouse bone marrow assay (12). Protein concentrations were measured at 278nm with a Gilford Model 2400 spectrophotometer using an extinction coefficient $E_{278}^{0.1\%} \approx 1.0$ determined by protein dry weight on total urinary proteins.

Denaturation and Renaturation: In Experiments 1-3, 1ml of a solution of human urinary erythropoietin (0.52mg/ml; 1092U/mg) in 50mM Tris-acetate, 5mM $CaCl_2$, pH 7.8 (Buffer A) was made 6M in urea (Ultrapure, Schwartz/Mann) by addition of 360mg of the crystalline solid. The solution was incubated for 30 min at 20°C to afford denaturation. The samples were then treated as follows. Experiment 1: sample dialyzed twice against 1L volumes of Buffer A, 4 hr each. Experiment 2: samples made 1% in sodium dodecyl sulfate (SDS), incubated 1 hr at 37°C, dialyzed sequentially for 4 hr against 1L volumes of Buffer A, 6M urea containing 1gm of Dowex AG-1x8; Buffer A, 1gm Dowex AG-1x8; Buffer A (13). Experiment 3: sample made 10mM in mercaptoethanol (MSH) and 1% in SDS, incubated 1 hr at 37°C, dialyzed sequentially against 1L volumes of Buffer A, 6M urea, 10mM MSH, 1gm Dowex AG-1x8; Buffer A, 6M urea, 1gm Dowex AG-1x8; Buffer A. In Experiment 4 1ml of the erythropoietin solution was made 10mM in MSH without other additions, incubated for 30 min at 20°C, and dialyzed twice against 1L volumes of Buffer A.

Polyacrylamide Gel Electrophoresis (PAGE): Each 2ml separating gel contained 10.5% acrylamide, 0.92% N,N'-methylenebisacrylamide, 1mg ammonium persulfate, 2µl TEMED (Bio-Rad), 720mg urea, 0.2mg SDS, 50mM Tris-HCl, pH 8.3. Running buffer consisted of 600mg Tris base, 2.88gm glycine, 1gm SDS per liter. Samples

Table 1: Denaturation and Renaturation of Erythropoietin

Experiment	Addition[a]	Erythropoietin Specific Activity, U/mg[b]		Recovery, %
		Before	After	
1	Urea	1092	1005	92
2	Urea/sodium dodecyl sulfate	1092	785	72
3	Urea/mercaptoethanol/ sodium dodecyl sulfate	1092	0	0
4	Mercaptoethanol	1092	1103	101

a) See Methods.

b) Measured by in vitro mouse bone marrow technic.

were made 6M in urea and 1% in SDS and incubated for 60 min at 20°C prior to electrophoresis at 3mA/gel for 3-4 hr. Molecular weight standards (Bio-Rad) were treated similarly. After electrophoresis the sample gel was sliced into 1mm sections, and the protein was eluted from each section by incubation in 1.0ml of Buffer A, 6M urea for 16 hr at 4°C. The gel eluates were dialyzed free of SDS and urea and assayed for erythropoietin activity. The gel containing the molecular weight markers was fixed in 50% methanol/10% acetic acid/40% water and stained with Coomassie Blue-R.

RESULTS AND DISCUSSION: Erythropoietin can be denatured and renatured with restoration of biological activity (Table 1). After denaturation with 6M urea dialysis to remove urea resulted in recovery of virtually all of the activity (Table 1, Experiment 1). Similarly, denaturation of erythropoietin with 6M urea and 1% SDS (Table 1, Experiment 2) and subsequent exhaustive dialysis resulted in a specific activity of 785U/mg, representing a 72% recovery. This degree of reversal of SDS denaturation compares quite favorably to that previously reported for a series of proteins (13). In contrast, denaturation of erythropoietin followed by reduction of disulfide bonds with MSH resulted in complete loss of activity without subsequent recovery (Table 1, Experiment 3) despite attempts to optimize conditions. This effect of MSH could be due to reduction of one or more inter-chain disulfide bonds or, alternatively, reduc-

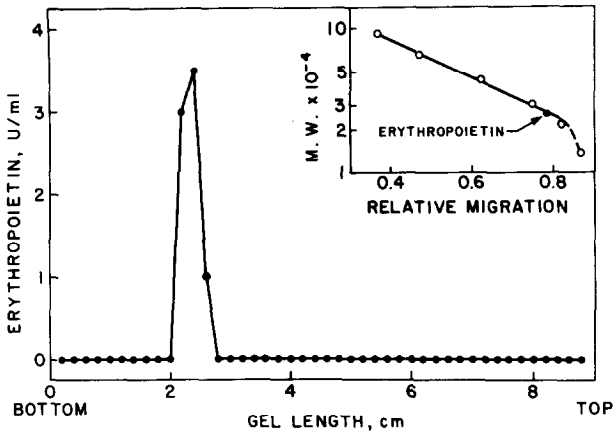

Figure 1. Molecular weight of human urinary erythropoietin (●) determined by polyacrylamide gel electrophoresis in 6M urea/0.1% sodium dodecyl sulfate. Two hundred micrograms of protein (87U/mg, 17U) were denatured in 6M urea, 1% sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis (9) (10.5% acrylamide). Upon completion, the gel was sliced and the erythropoietin was eluted, renatured and assayed (see Materials and Methods). Insert: molecular weight standards (Bio-Rad) (○) from upper left to lower right: phosphorylase B subunit, 94,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,000.

tion of (an) intra-chain bond(s) critical to the tertiary structure and, hence, biological activity of the hormone. Importantly, addition of MSH to erythropoietin without prior denaturation (Table 1, Experiment 4) did not result in a loss of activity, strongly suggesting that the critical disulfide bridge(s) is (are) relatively inaccessible to solvent, i.e., "buried" in the erythropoietin molecule.

These results indicated that a determination of the hormone's molecular weight under denaturing conditions was feasible. Although disulfide reduction could not be employed (see above) others have shown that reasonable molecular weight estimates can be achieved with intact disulfide bonds (14). Accordingly, 200μg of erythropoietin (87U/mg) were denatured in 6M urea/1% SDS and subjected to PAGE. After elution and renaturation a single peak of erythropoietin activity was found (Fig. 1). A plot of the relative mobilities of the protein standards versus log molecular weight is linear, and extrapolation from the mobility of the erythropoietin peak (0.77) yields an apparent molecular weight of 25,000 (Fig. 1, insert). Additional experiments using higher concentrations

of acrylamide in the gel did not result in a significant lowering of this value, indicating that the "minimal asymptotic molecular weight" for this glycoprotein was achieved (15,16).

The value obtained in the present study for the molecular weight of erythropoietin, 25,000, places the hormone on a par with several other polypeptide hormones, notably growth hormone (21,500) (17), thyroid stimulating hormone (25,000) (18) and luteinizing hormone (30,000) (19), but it is substantially lower than several other recent estimates (2,3,20,21). Goldwasser concluded that the molecular weight of erythropoietin from sheep plasma is 46,000 using PAGE in SDS (22). This was determined with iodinated, biologically inactive material, and conditions required for the "minimum molecular weight" of the glycoprotein were not detailed (see below). It is unlikely that this apparent discrepancy between the molecular weights of the plasma and urine hormones is due to a species difference, since, among other mammalian polypeptide hormones studied thus far, there is rarely a significant interspecies variation. It is possible that the hormone is subjected to proteolytic cleavage or sugar hydrolysis on its passage through the kidney, thus explaining the lower molecular weight for the urinary hormone. However, such "processing" might be presumed to result in substantial heterogeneity of size among biologically active fragments, not the single 25,000 molecular weight molecule observed in the present study. Rather, the difference probably lies in the assumptions inherent in the methods used to estimate molecular weight. The ultracentrifugation and gel filtration methods employed previously were performed upon samples of native, undenatured erythropoietin and, as such, require that average hydrodynamic properties or globular conformation of proteins be assumed. Furthermore, the samples were not shown to be homogeneous erythropoietin and, hence, could be plagued by phenomena of protein-protein interaction and aggregation which many proteins, including erythropoietin (12), can exhibit. Interestingly, the early studies of Rosse *et al.* using radiation inactivation resulted in a molec-

ular weight estimate of 27,000 for erythropoietin (1), in close agreement with the present study.

The method of PAGE under denaturing conditions of 6M urea and 0.1% SDS employed here obviates the problem of protein-protein interaction, and since biological activity was used to identify the hormone, neither strict homogeneity nor a high concentration of protein was required. It is important to make note of the potential for artifactually high molecular weight estimations which may result when this method is applied to glycoproteins (15,16). Care must be taken to employ sufficiently high acrylamide concentrations, thereby maximizing the role of size and lessening the role of charge in the method. In so doing, a reliable "minimum asymptotic molecular weight" is derived. Such precautions were taken in this study.

These results serve as a basis for further studies of the biochemistry of erythropoietin and illustrate a general approach to the examination of polypeptide hormones available in severely restricted quantities.

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