EXHIBIT 3

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A Probable Conformational Difference Between Recombinant and Urinary Erythropoietins

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ABSTRACT **Urinary and recombinant human erythropoietin differ with respect to ease of iodination, inactivation by iodination, second derivative and circular dichroic spectra, rate of inactivation by trypsin and glycosylation pattern. All of these differences are compatible with a significant difference in conformation of these two forms of erythropoietin. Proteins 28:94–98, 1997 © 1997 Wiley-Liss, Inc.**

Key words: cytokines; glycoproteins; iodination; protein modification; tyrosine environment

INTRODUCTION

The glycoprotein erythropoietin (epo) is the primary regulator of red cell formation in mammals. It is secreted from the kidneys constitutively and in greatly increased quantity under conditions of hypoxic stress. Naturally occurring epo, isolated from the urine of severely anemic patients (u-epo)[1] has been partially characterized as has been recombinant human epo (r-epo) expressed by Chinese hamster ovary cells transfected with the human epo gene.[2] The former was reported to have a lower potency (units** per milligram of protein)[1] (80,000–90,000) than does r-epo (about 130,000),[2] although they both have the same amino acid sequence. They have been reported to have essentially the same amount of carbohydrate with oligosaccharide structures that do not differ to any great extent.[3,4] Very little is known yet, concerning the structural basis for the biological effect of epo or any possible structural basis for the observed difference in potency between the two preparations.

In the present paper we demonstrate differences between u—epo (the β form[1]) and r-epo† with respect to ease of iodination, and to inactivation by iodine. We also present additional data, indicating an apparent difference in carbohydrate compositions and a probable conformational difference between u-epo and r-epo, which may account for the difference in potencies.

MATERIALS AND METHODS

Two different assay methods were used during the period of these studies. For the studies of epo activity after iodination, we used the in vitro rat bone marrow cell assay developed in this laboratory.[5] In later studies of trypsin inactivation we used a cell proliferation assay: ^{3}H-thymidine incorporation by UT-7-epo cells.[6] Both methods are based on standard curves using human recombinant epo previously standardized.[2]

Since the marrow cell assay has already been described, only the cell proliferation assay is detailed here. Assays were done with UT-7-epo cells (generously supplied by Amgen, Inc.) grown in 90% Iscove's modified Dulbecco's medium containing 2 mM glutamine and 10% fetal calf serum, passed no more than 3–4 days before being used. Cells were collected, washed with sterile PBS, and diluted to 10^5/ml in 96% RPMI 1640, containing 2 mM glutamine and 4% fetal calf serum. Aliquots (0.1 ml) were put into wells of Nunclon 96 well trays; 0.1 ml of sample or of standard epo was added and the trays incubated at 37°C in 5% CO_2: 95% air for 23 hours. To each well, 0.05 ml of ^{3}H-thymidine (0.05 μCi; specific activity 83.3 mCi/mg) in the same medium were added. The cells were returned to the incubator for 1 hour and then harvested by using a PhD cell harvester (Cambridge Technology, Inc.) on Whatman glass microfiber/quartz (Grade 934-AH) filters that had been prewet with water. The cells were washed five times with water, with 95% alcohol and dried at 80°C for 15 minutes in a vacuum oven. When cool they were put into 4 ml of Biosafe II fluor (Research Products International, Mount Prospect, IL) and counted. The standard curves (1.0–8.0 mU (0.255–2.04 pmol) per well, 6–8 replicates per sample) were fit by using Systat programs, and the resulting regression equation was used to determine titers of the experimental samples. The regression equation correlation coefficients for this assay was 0.996.

**One unit of epo activity is defined as the biological activity contained in 0.29 mg of the Second International Reference Preparation. This is equivalent to 7.75 ng of recombinant human epo or 0.255 pmol.

†In this paper r-epo refers only to that expressed by Chinese hamster ovary cells. Other types of r-epo have other properties.

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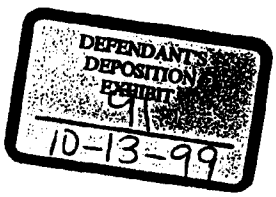


Because of the scarcity of starting material the u-epo used in these experiments was that originally prepared,[1] which had retained its original specific activity of 82,700 U/AU when it was first purified.[1] When these experiments were done, the same preparation was assayed to have a specific activity of 88,700 U/AU. The r-epo was a gift from Amgen Inc. and was derived from Chinese hamster ovary cells carrying an amplified human epo gene. It was purified by the method described by Lai and Strickland.[7]

Iodination was done by the method of Fraker and Speck.[8] Spectroscopy was performed with a Perkin-Elmer Lambda 4B instrument by using enhanced scan operating software, and the second derivative spectra analyzed by the method of Ragone and coworkers[9] CD spectra were done on a Jasco model J-600. For the wavelength region 190–250 nm, we used cuvettes with 0.1-cm light path with epo in 2 mM phosphate at pH 7. The A_{280} for r-epo was 0.165 and for u-epo was 0.167. For the wavelength range 250–320 nm we used 1-cm light path cuvettes and the same buffer. The A_{280} for r-epo was 0.271 and for u-epo was 0.275. The spectra in Figure 3 represent one of four analyses with essentially the same results.

Trypsin hydrolysis experiments were done as follows: 20μl of epo (A_{280} = 0.254 for both r- and u-epo) in 2 mM phosphate buffer pH 7.2 were added to a PEG-coated V-vial. Zero time aliquots (2μl) were removed and 6μl of a 1.1 ng/ml solution of TPCK trypsin in 0.1 M Tris buffer, 0.01 M $CaCl_2$ plus 0.02% Tween 20 pH 8 added to vials preequilibrated at 37°C. Aliquots (2μl) were removed at 1, 3, and 5 minutes, added to 5μl of a solution of soybean trypsin inhibitor (2 ng/ml in 0.1 M phosphate buffer pH 6.5 plus 0.02% Tween 20) followed by 1.0 ml of cold PBS containing 0.1% BSA. The samples were then further diluted for assay. The zero time activities were 4.9 U/ml for r-epo and 6.5 U/ml for u-epo.

Gel electrophoresis was done according to Laemmli.[10] The protein samples (50-200 ng/l) were denatured by heating at 100°C for 3 minutes in sample buffer [2% (w/v) SDS, 0.0625 M Tris pH 6.8, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.0375% (w/v) bromphenol blue]. After cooling to room temperature 1–4 μl (50 mg/μl) per well of the denatured sample were loaded and run in a discontinuous polyacrylamide gel consisting of a separating (lower) gel (15% polyacrylamide) and a stacking (upper) gel (4.5% polyacrylamide) and in the discontinuous buffer system of Laemmli.[10] The gel size was 0.5 mm × 7 cm (*l*) × 8 cm (*w*). A constant voltage gradient of 15–20 V/cm was applied. Routinely, Bio-Rad low range SDS-PAGE molecular weight standards (cat. no. 161-0304) were run on the same gel as the test samples.

Iodogen was bought from Pierce Chemical (Rockford, IL), $^{59}FeCl_2$, $Na^{125}I$, and ^3H-thymidine from Amersham Corp. (Arlington Heights, IL), fetal calf serum, which was heated at 56°C for 30 minutes, and NCTC 109 from GIBCO/BRL (Grand Island, NY), and rat serum from Pel-Freeze Biological (Roger, AR), Long-Evans rats from Charles River (Wilmington, MA), sialidase, *N*-glycanase, *O*-glycanase, and RPMI 1640 from Sigma Chemicals, TPCK trypsin from Worthington and soybean trypsin inhibitor from Boehringer Mannheim. Supplies for SDS-PAGE were bought from Biorad (Hercules, CA), as were the molecular weight markers.

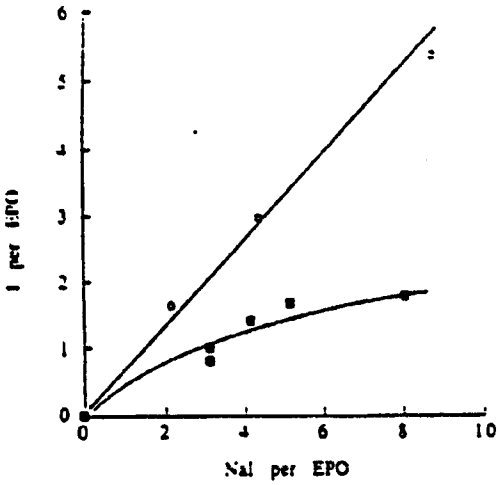


Fig 1. Iodination of recombinant and urinary erythropoietin. The conditions of iodination of one typical experiment are in the text. The open circles represent r-epo; the filled squares represent u-epo.

RESULTS

The data in Figure 1 were derived from an experiment in which the $Na^{125}I$/epo molar ratio in the reaction mixture was varied and the amount of iodine substitution determined. The data show a marked difference in ease of iodination between r-epo and u-epo. With u-epo, substitution was less than 2 atoms of I per molecule of epo when the molar ratio of NaI/epo in the reaction mixture was 8, but for r-epo at the same NaI/epo ratio, there were more than 4 atoms of I per epo molecule. These findings suggest that the accessibility of iodinatable residues is significantly lower for u-epo than for r-epo. We have found that the only residues in epo that are iodinated under these conditions are tyrosines.

Biological activity of the two epo preparations, as measured in vitro by marrow cell hemoglobin synthesis, was also differentially affected by iodination; r-epo is inactivated to a markedly lesser extent than is u-epo. As can be seen (Fig. 2), substitution of one to two I atoms per u-epo molecule is sufficient to cause complete loss of activity, but for r-epo the inactivation curve indicates little or no loss of activity at one

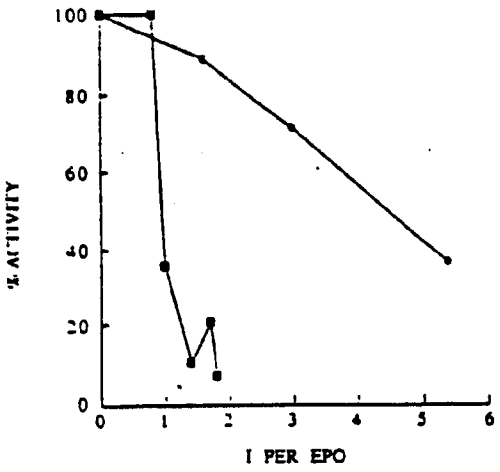


Fig. 2. Inactivation of erythropoietin by iodination. Activity was measured by the bone marrow cell method[9] at three concentrations per sample and five replicates for each. The open circles represent r-epo; the filled squares represent u-epo. The starting concentration (100%) were 1.2 U/ml for r-epo and 1.6 U/ml for U-epo.

I/epo, about 30% inactivation at three I/epo, and extrapolates to zero activity at about eight atoms per molecule. We interpret these results as additional evidence of a difference in conformation of the two epo preparations resulting in different degrees of accessibility of critical tyrosyl residues, and of the role of conformation in biological activity.

Support for this interpretation is given by study of second-derivative absorption spectroscopy. As the data in Table I show there is a significant ($P < 0.01$) difference in the a/b ratio[9] between the two forms reflecting a difference in the environment of the tyrosine residues. The values of the a/b ratio for r- and u-epo in the denatured state, in 6 M guanidine, are the same (1.03). From these ratios, the estimate of the number of tyrosine residues exposed to the solvent shows a small but significant difference between r-epo and u-epo (Table I). Circular dichroism spectra of u-and r-epo at the same concentrations showed a clear difference between the two. The difference in the region 195–230 nm suggests a difference in secondary structure (Fig. 3A) and that around 280–290 nm suggests a difference in the environment of the tyrosine residues (Fig. 3B).

In another test of possible conformational difference between the two epos we studied the rates of inactivation by a very low concentration of trypsin. The results (Fig. 4) show a very clear and consistent difference: u-epo has a half-life, under these conditions, of about 1 minute. By contrast, the half-life of r-epo is greater than 4 minutes.

Another set of differences between r-epo and u-epo is seen in the experimental results summarized in

TABLE I. Interpretation of Second Derivative Spectra

	a/b native	a/b denatured	Tyrosines exposed to solvent
u-epo	0.77 ± 0.02	1.03 ± 0.05	2.6
r-epo	0.85 ± 0.01	1.03 ± 0.02	3.0

The ratios are expressed as means ± S.D. with n = 3 for r-epo and 4 for u-epo.

Table II. The two forms of epo were sequentially treated with sialidase, *O*-glycanase and *N*-glycanase and apparent molecular weights determined on a reducing, 15% polyacrylamide gel in the presence of SDS. We found significant differences in M_r between native r- and u-epo samples, with r-epo being about 1.2 kDa greater in M_r than u-epo. Sialidase caused a decrease in M_r of 3.4 kDa for r-epo and 4.1 kDa for u-epo. *O*-Glycanase treatment resulted in loss of 2.9 kDa for r-epo and 1.0 kDa for u-epo, while treatment with *N*-glycanase caused equal changes in M_r with the final value (21.6 kDa) being the same for both samples.

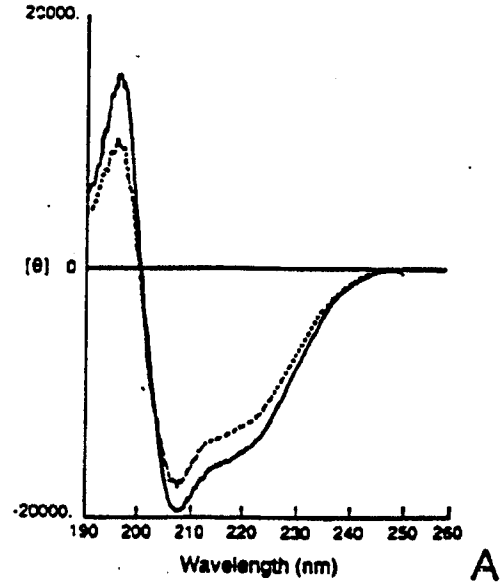
DISCUSSION

We have presented data strongly suggestive of different conformational states for u- and r-epo. The greater accessibility of tyrosines in r-epo to iodination (Fig. 1) agrees with the difference in second derivative spectra between the two epo preparations. Our findings that u-epo is much more easily inactivated by iodine substitution (Fig. 2) suggest that a critical tyrosine is somewhat masked in r-epo but is more accessible in u-epo.

The differential susceptibility to hydrolysis by trypsin (Fig. 4) also suggests a difference in higher order structure between u-epo and r-epo, in agreement with the circular dichroic spectroscopic data (Fig. 3). Similar CD spectra have been published,[11,12] but in neither paper was there a direct comparison of u- and r-epo by this method. In addition, the CD spectra reported were determined in different buffers and at different concentrations.

It seemed plausible that the differences we observed might have been due to the exposure of u-epo to phenol in the original purification method[3] when it may have been unfolded and, after being returned to aqueous solution, refolded to a somewhat different conformation with lower activity. Since r-epo similarly treated with phenol and purified by the same method used for u-epo has the same potency as does r-epo not so treated (T. Strickland and J. Egrie, personal communication), exposure to phenol was clearly not the basis for the difference in potency between the two forms.

Because the log M_r vs distance plot for marker proteins on a denaturing, reducing SDS gel is not



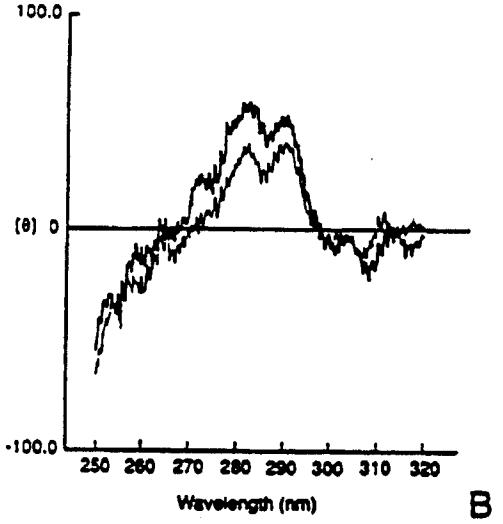


Fig. 3. Circular dichroic spectra of urinary and recombinant erythropoietin. The broken line represents u-epo; the unbroken line represents r-epo. This represents one of four separate analyses. A: Wavelength range 190–250 nm. B: The range is 250–315 nm.

linear and bends at about the molecular size of epo, and because of the sensitivity of mobility to small changes in gel composition, it is difficult to get precise and consistent values for M_r by this method. In our hands the apparent M_r for r-epo varies between 32,000 and 38,000; in this run it was at the high end of the range. The differences due to enzymic

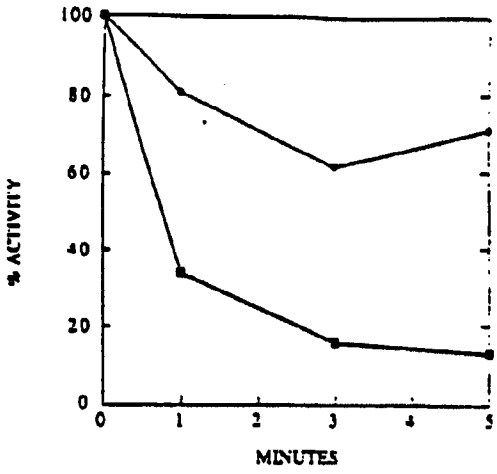


Fig. 4. Effect of limited trypsin hydrolysis on erythropoietin activity. Each point was derived from 6 to 8 replicates each at two different levels. Unitage was determined from the regression equation of the standard curves. The open circles represent r-epo; the filled squares represent u-epo. The zero time concentration of r-epo was 3.8 mU/ml and that of u-epo was 5.9 mU/ml.

TABLE II. Effects of Removal of Carbohydrate on Apparent Molecular Weights of Recombinant and Urinary Erythropoietin

	r-epo		u-epo	
	M_r kd	ΔM_r kd	M_r kd	ΔM_r kd
Native	38.2	—	37.0	—
Asialo	34.8	3.4	32.9	4.1
O-glycanase	31.9	2.9	31.9	1.0
N-glycanase	21.6	10.3	21.6	10.3

ΔM_r refers to the difference in apparent molecular weights after treatment with each enzyme.

hydrolysis, however, are consistent when compared with each other and with native epo on the same gel.

The data in Table II suggest a difference in *O*-linked carbohydrate and possibly in sialic acid content with u-epo having less *O*-linked oligosaccharide and more sialic acid than r-epo in agreement with other reported values.[3,4] The differences we find in conformation may be due to an effect of the difference in carbohydrate composition on folding of the glycoprotein. A similar study of u- and r-epo expressed by COS cells, rather than CHO cells, showed essentially no difference between the two.[13] The possibility that the different cell types do not glycosylate the epo protein identically is very real, especially in view of observations with other expression systems.[14–17] Our data probably differ from those presented earlier[4] because of different preparations of u-epo studied.

CONCLUSION

The data strongly suggest a significant conformational difference between recombinant and urinary erythropoietins. This difference is especially apparent in the molecular environment of one or two of the tyrosine residues and in the sensitivity to tryptic hydrolysis. It may also account for the difference in biological activity found for these two preparations.

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