Erythropoietin Structure-Function Relationships
IDENTIFICATION OF FUNCTIONALLY IMPORTANT DOMAINS*

(Received for publication, April 22, 1994, and in revised form, June 10, 1994)

Danyi Wen†, Jean-Paul Boissel‡, Mark Shower$, Baird C. Ruch, and H. Franklin Bunn§
From the Hematology/Onology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115.

In order to delineate functionally important domains in erythropoietin (Epo), we have prepared and tested a series of amino acid replacements at 51 conserved sites predicted to be on the surface of the molecule. Alanine replacements permitted preservation of α-helical structure. Wild type and mutant Epo cDNAs were transiently expressed at high levels in COS1 and COS7 cells. The biological activity of wild type and mutant Epos was assessed in three Epo-responsive cell types: primary murine erythroid spleen cells, the murine HCD57 erythroleukemia cell line, and the human UT7-EPO leukemia cell line. When Arg→Ala on predicted Helix A was replaced by Ala, biological activity was substantially reduced, whereas replacement with Glu resulted in total loss of specific bioactivity. In a similar manner, the mutein Arg→Ala in Helix C was completely lacking in biological activity, whereas both Ser→Ala and Leu→Ala had decreased bioactivity. In Helix D, the mutein Gly→Ala had markedly decreased bioactivity, whereas that of the adjacent Lys→Ala mutein was moderately impaired. In contrast, Ala replacements at three nearby sites on Helix D (147, 146, and 143) resulted in muteins with increased bioactivity. In conclusion, our mutagenesis experiments have identified functionally important domains on the surface of the Epo molecule, at sites comparable with those established for other cytokines.

Erythropoietin (Epo) is a 30.4-kDa glycoprotein hormone that regulates red blood cell production (1, 2). Biochemical studies (3, 4) led to molecular cloning of the Epo gene (5, 6). High level expression has enabled recombinant human Epo to be used very effectively in the treatment of anemias. Moreover, the experimental use of recombinant Epo has greatly advanced our understanding of the molecular mechanisms underlying erythropoiesis.

Epo is a member of an extensive cytokine family which also includes growth hormone, prolactin, interleukins 2 through 6, G-CSF, GM-CSF, M-CSF, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor (7–9). Although sequence homology is weak, genes encoding these proteins have similar numbers of exons and share a clear relationship between intron-exon boundaries and secondary structure (9). All of these cytokines are predicted to fold into a common compact globular structure, consisting of four amphipathic α-helical bundles. Such theoretical models of the structures of human growth hormone (HGH) (10) and IL-4 (11) have been in good agreement with the subsequent structures established by x-ray diffraction (HGH) (12, 13) or by multidimensional NMR (IL-4) (14, 15). Moreover, the crystal structures of GM-CSF (16, 17), G-CSF (18), monomeric M-CSF (19), IL-2 (20, 21), and IL-5 (22) are also in reasonable agreement with their predicted structures (for review, see Ref. 23).

Epo’s high carbohydrate content (39%) and the heterogeneity of these carbohydrate chains have thus far precluded the determination of its three-dimensional structure by either x-ray diffraction or by NMR. In order to gain an understanding of Epo structure-function relationships, we first determined the coding sequences of Epo from six mammals representing five different orders, thereby identifying regions of high homology that are likely to have structural and/or functional importance (24). We then developed a computer-generated model of Epo’s three-dimensional structure and have tested it by means of a series of scanning deletion muteins, which fully support a four α-helical bundle structure (8, 25) in common with that of the other cytokines mentioned above. As described in this report, we have exploited this information in the design and testing of single amino acid replacement muteins that provide information on functionally important domains of human Epo.

MATERIALS AND METHODS
Construction of Wild Type and Mutant Mammalian Expression Plasmids—cAHEPO FL12, kindly provided by Genetics Institute (Cambridge, MA) (5), is an M13 plasmid, containing a 1.4-kilobase pair EcoRI-EcoRI human Epo cDNA insert. A 943-base pair EcoRI-BgIII fragment, which includes the complete coding sequence of the wild type human erythropoietin as well as untranslated regions 216 base pairs upstream and 183 base pairs downstream, was inserted into the mammalian expression plasmid pSG5 (Stratagene) (26) and designated pHG5-EPO/WT. Site-directed Mutagenesis—Site-directed mutagenesis was carried out by a modification of the protocol described by Kunkel et al. (27). For further details see Ref. 25. Since a 40–60% mutation yield is normally obtained, four to five double-stranded DNA clones from each reaction were sequenced with 7-deaza-dGTP and Sequenase (U. S. Biochemical Corp.) (28) to verify the mutation. In addition, each mutant was screened by restriction mapping or, less commonly, by full sequencing in order to detect the presence of additional unwanted mutations.

Production of Wild Type and Epo Muteins in Mammalian Cells—COS 1 or COS7 cells, grown to 40–60% confluence, were transfected with 20 μg of recombinant plasmid DNA10-cm dish using the calcium phosphate precipitation protocol (29). As a control of transfection efficiency, in several experiments 2 μg of pCH110 plasmid (Pharmacia Biotech Inc.) was co-transfected and β-galactosidase activity was measured in the cytoplasmic extracts.

Quantitation of Transiently Expressed Recombinant Epos—The amount of secreted protein in the supernatants of transfected COST
Functionally Important Domains of Human Epo

Our objective is to identify the functionally important domains of the Epo molecule by preparing muteins that have altered specific bioactivity, i.e., significantly higher or lower biological activity per unit mass of protein. Such muteins must satisfy two criteria: 1) efficient secretion by COS cell supernatants and 2) preservation of the overall folding and three-dimensional structure, thereby allowing full recognition (with normal or nearly normal binding affinity) by the polyclonal anti-Epo antisera that we use in our radioimmunoassay. In order to satisfy these criteria, our strategy employed the use of muteins that have only single amino acid replacements. For all but a few muteins, we have made alanine substitutions in order to preserve a helical structure (36). A comprehensive alanine replacement scan of Epo would require the preparation and testing of nearly 166 muteins. Such an undertaking would be prohibitively labor intensive and costly. We therefore devised a strategy to reduce the number of required replacements, by applying the information we recently obtained and reported on mammalian Epo sequences (24), as well as on scanning deletion muteins (25).

On the basis of its primary amino acid sequence and disulfide bonds, Epo is predicted to have a four-antiparallel amphipathic α-helical bundle structure (8, 25) in common with other members of the cytokine family (9). We have shown that deletions in nonhelical regions at the N terminus, the C terminus, and in the loops between helices result in the formation of Epo proteins that are readily secreted from the cell and are biologically active (25). These regions (Fig. 1) can be ruled out as domains essential for function, such as the sites involved in the binding of Epo to its receptor. Moreover, residues that are not conserved among mammals (Ref. 24 and Fig. 1) are also unlikely to be functionally important sites. Our previous scanning deletion analysis (25) suggests that the functionally important receptor contact sites are likely to reside on the predicted α helices. Furthermore, since the amphipathic helices are predicted to bind to one another along their hydrophobic surfaces, the biologically relevant contact sites are likely to be residues predicted to be on the external surfaces of the helices (25). These considerations greatly restricted the number of mutations needed to define critical functional domains of Epo and focused our analysis on the conserved surface residues of the four helices as well as residues adjoining the helices. In addition to the muteins which were selected according to the above criteria, we prepared 3 alanine replacement muteins at conserved sites predicted to be in the hydrophobic interior: Trp64, Ser71, and Thr106. As expected (and shown in Table I), all of these muteins had normal specific bioactivity.

Fig. 1 depicts the residues excluded on the basis of the three above-mentioned criteria as well as the 56 replacement muteins we have prepared and tested.2 Table I presents specific bioactivity data on all of these muteins tested in three Epo-responsive cell types. In general there was excellent agreement

---

2 53 muteins at 51 predicted external sites.
between the three cell bioassay systems. As discussed below, a few muteins displayed different biological activity depending on the species of the assay cell, specifically the human UT7 versus the murine HCD57 and spleen cells.

It should be noted that in a few of these dose-response curves, at the highest concentrations of Epo tested, there was a drop-off in the incorporation of [3H]thymidine (for example, see Figs. 4B and 5C). This is due to the fact that high levels of biologically active Epo cause terminal maturation and cessation of cell division. Because of this possibly confounding phenomenon, it is important to have, as in the experiments that we report here, a full dose-response curve for each mutein over a wide (50-fold) concentration range.

At three sites in human Epo, we found a marked decrease in specific bioactivity. (a) In predicted Helix A, replacement of Arg14 by Ala resulted in considerably impaired biological activity, particularly when assayed by the two mouse cell systems (Fig. 2). In order to further delineate residue 14 as a functionally important site, the following additional muteins were prepared and tested: Arg14→Leu and Arg14→Glu. As shown in Fig. 2, R14L had normal specific bioactivity, whereas R14E was nearly inactive, as determined by all three cell bioassays. The differential bioactivity of these Arg14 replacementmuteins with alanine, leucine, or glutamic acid indicates a precise stereochemical integrity at this site that is essential for Epo’s function. When other external sites predicted to be on Helix A were replaced by Ala (Ser5, Arg16, Glu17, Leu41, Glu43, and Glu46) normal bioactivity was observed. Particularly noteworthy is the lack of abnormal function when the 3 Glu residues were replaced. An acidic residue in Helix A is thought to be critical for the binding of IL-2, IL-3, and GM-CSF to their respective receptors (23).

(b) In predicted Helix C, the Arg103→Ala mutein was completely devoid of specific bioactivity in all three bioassay systems (Fig. 3A). These results confirm and extend recent work of Grodberg et al. (37) who employed only the mouse spleen cell assay (32). Replacement of the adjacent residue Ser104 with Ala resulted in normal bioactivity when tested in human UT7 cells, but low (35%) bioactivity when tested in mouse HCD57 cells (Fig. 3B). In a similar manner, as shown in Fig. 4C, alanine replacement of Leu106 in the CD loop resulted in a mutein which had slightly enhanced bioactivity (158%) when tested in the human cells, but low activity when tested in mouse HCD57 cells (48%) or in mouse spleen cells (32%). These results indicate that this region of predicted Helix C contributes strongly to Epo’s function. In contrast, Ala replacement of other conserved residues predicted to be on the surface of Helix C (Gln82, Leu81, Lys87, Ser103, Gly106, and Thr106) generated muteins with normal Epo bioactivity. His44→Ala and Asp56→Ala were not secreted from COS 7 cells.

(c) In predicted Helix D, the Gly151→Ala mutein was also nearly devoid of specific bioactivity, as assayed in both human UT7 cells and murine HCD57 cells. Moreover, the Ala replacement of the neighboring residue Lys152 had decreased specific bioactivity in the two murine cell bioassays but normal activity in human UT7 cells. Of additional interest is the increased specific bioactivity noted in muteins in which nearby residues were replaced by Ala: Asn147, Ser148, Arg149, and perhaps Lys149 (Table I). In contrast no significant abnormalities in specific bioactivity was noted when Ala replaced the following residues in Helix D: Asp136, Arg39, and Arg150. Because the exact C-terminal boundary of Helix D is uncertain, and because preliminary data from other laboratories (38, 39) suggest that the C-terminal region of Epo may be functionally important, we
Functionally Important Domains of Human Epo

Fig. 2. Bioassays of mutations at Arg¹⁴ (A, Arg¹⁴ → Ala; B, Arg¹⁴ → Glu; and C, Arg¹⁴ → Leu). Epo-dependent HCD57, UT7, and primary murine spleen cells were incubated with increasing amounts of wild type (WT) or mutant Epos (muteins). In this, as in subsequent figures, the number after WT refers to a particular transfection. WT ave refers to the average of three separate COS cell transfections. The concentrations of wild type Epo and Epo muteins were measured by radioimmunoassay. The uptake of [³H]thymidine provides a measure of Epo-induced cellular proliferation. Differences in incorporation of radioactivity as shown in the ordinate reflect different doubling times of the three cell types. The concentration of Epo in the COS cell supernatants is determined by an Epo radioimmunoassay (see "Materials and Methods"). In the bioassay curves the abscissas represent increasing concentrations of wild type or mutant Epo. Each assay consisted of three replicate measurements of [³H]thymidine incorporation at nine concentrations of Epo. The area under the curve (i.e., the sum of these values, corrected for nonspecific background incorporation) provides the measure of specific bioactivity used in the compilation of Table I.

tested alanine replacements at positions Lys¹⁵², Leu¹⁵³, Lys¹⁵⁴, Leu¹⁵⁵, Tyr¹⁵⁶, Thr¹⁵⁷, Gly¹⁵⁸, and Glu¹⁵⁹ which are predicted to be in the adjacent nonhelical segment at the C terminus. As shown in Table I, Lys¹⁵⁴ → Ala had increased bioactivity, whereas the other replacements were indistinguishable from wild type.

We examined competition between wild type and mutein Epos as shown in Fig. 5. A bioassay in UT7 and in HCD57 cells was performed with a constant amount of wild type Epo and increasing amounts of competitor mutein Epo. Even when present at 5-fold excess, the biologically inactive Arg¹⁰³ → Ala competitor Epo mutein failed to affect the specific biological activity of wild type Epo, in either cell system (Fig. 5A). In comparison, a 5-fold excess of the Arg¹⁴ → Glu mutein resulted in a slight enhancement of bioactivity, suggesting that, at high concentrations, it can bind to the receptor and induce proliferation. As a control (Fig. 5C), the Arg¹⁴ → Leu mutein, which has nearly normal specific bioactivity, when added in 5-fold excess, gave the expected robust increase in proliferation.

At other sites predicted to be on Epo's surface, Ala replacement had no significant effect on biological activity. Lys⁴⁵ → Ala in interhelical region A-B resulted in a mutein with wild type Epo bioactivity. Serine substitutions at Phe⁶⁸, Ala⁵⁰, Trp⁵¹, and Lys⁶² also resulted in muteins with wild type Epo bioactivity, whereas the Tyr⁴⁹ → Ser mutein had 150% of wild type Epo bioactivity in both UT7 and HCD57 cells. As shown on Table I, Ala replacements of conserved residues predicted to be on the surface of Helix B (Glu⁴⁹, Glu⁶¹, Trp⁶¹, Glu⁶₂, Gly⁶₅, Leu⁶⁹, Ser⁷¹, and Arg⁶⁰) had normal specific bioactivity. Ala⁷³ → Gly had increased bioactivity in UT7 cells. Glu⁷² → Ala was not secreted by COS cells as determined by radioimmunoassay and Western blot analysis (results not shown) and therefore could not be assayed for bioactivity.

DISCUSSION

Initial studies of Epo's structure-function relationships relied primarily on the use of antibodies (40-49). Mapping of specific domains has been based on polyclonal antibodies raised against Epo peptides and monoclonal antibodies raised against either peptides or the intact protein. However, it cannot be assumed that antibodies which neutralize Epo's biological activity always bind to a functionally important domain such as the receptor binding site. Antibodies that bind to a functionally irrelevant site may impair bioactivity by either steric hindrance or the induction of a conformational shift. Reports that antibodies to peptide 111-129 (42, 48) and to the C-terminal...
peptide 152–166 (47) neutralize Epo function, must be viewed with caution in view of our demonstration that deletions at these sites: Δ110–119, Δ163–166 (25) and Ala replacements at residues 152–159 (Table I) fail to affect biological activity.

In contrast, several (40, 41, 45, 47), but not all (43), investigators have reported that biological activity is not impaired when Epo is bound to antibodies directed against N-terminal peptides. These results must be reconciled with our finding that replacement of Arg14 with Ala or Glu, diminishes or abolishes Epo biological activity, respectively. Arg14 is predicted to be the fourth residue in α-Helix C that might bind an epitope C-terminal to Arg14.

There is much less information in the literature on the preparation and testing of site-directed mutants of Epo. Initially, attention focused on the disulfide bonds (50) (see also Ref. 25) and N-linked glycosylation sites (51). Quelle et al. (38) prepared muteins in which 6–8-residue cassettes were inserted into the N and C termini of Epo. The former had normal specific bioactivities in agreement with our recent data (25, 52), showing nearly normal specific activity of Epo in which residues 2–5 were deleted and of Epo containing the propeptide at the N terminus. The cassettes which Quelle et al. (38) inserted into the C terminus resulted in muteins that were biologically inactive. In contrast, as mentioned above, we have shown that muteins containing deletions of the 4 C-terminal residues (Δ163–166) or replacement of those amino acids by a Lys-Asp-Glu-Lys (KDEL) or polyhistidine sequence, all had full specific bioactivity (25).

Immunologic studies led Sytkowski and his colleagues (42, 48) to conclude that a functional domain lay within residues 99–129. They then prepared a series of muteins in which Glu-Phe was inserted in place of sequential deletions of 3 adjacent amino acids (53). Muteins in the region 99–110 were not secreted from COS7 cells and, when transcribed and translated in vitro, were not biologically active. Recently, this group has reported on Ala replacements of residues 100–109. In full agreement with our results, they found that, with the mouse spleen cell assay, Arg103 → Ala was devoid of biological activity, whereas Ser104 → Ala and Leu105 → Ala had decreased bioactivity. In contrast, our earlier (25) and current results differ somewhat from the recent report of Bittorf et al. (39) on four deletion muteins and four Ala replacement muteins. They obtained the following specific bioactivities: Arg103 → Ala, 47%; Thr106 → Ala, 41%; Lys104 → Ala, 9%; and Glu105 → Ala, 26%, whereas we obtained values of 5, 114, 160, and 86%, respectively, for these muteins. Equally surprising is their finding that Δ13–17, predicted to be in Helix A, was secreted and had normal biological activity, whereas we found that Δ12–16 was not secreted and devoid of bioactivity (25) and that replace-
Fig. 4. Bioassays on mutations in Helix D that have abnormal function. Gly^{151} → Ala (A) and Asn^{147} → Ala (B) were assayed in the two cell types as described in the legend to Fig. 2.

Fig. 5. Bioassays of mixtures of Epo muteins with wild type Epo. Each panel shows Epo wild type alone (open squares), Epo mutein alone (solid triangles), and wild type Epo mixed with a 5-fold excess of mutein (open circles). In these mixtures, the abscissa refers only to the amount of wild type Epo in the assay. A, Arg^{103} → Ala; B, Arg^{14} → Glu; C, Arg^{14} → Leu.

ments of Arg^{14} markedly impair bioactivity. These investigators (39) employed only the human Epo-sensitive cell line TF-1, which in our hands is unstable and an unreliable assay system for muteins.

Our strategy for designing replacement muteins for studying Epo's structure-function relationships is based a three-dimensional model, reinforced by scanning deletion mutein experiments (25) and mammalian primary sequences (24). As summarized in Table I, we have tested mutations at 54 residues selected by the exclusion criteria described above. The reproducibility, validity, and interpretability of these experiments has been considerably strengthened by the use of three bioas-
say systems, a human cell line, a mouse cell line, and mouse spleen cells.

A number of the muteins that we have tested (R14A, K20A, A73G, S104A, L108A, K140A, R143A, S146A, N147A, K152A) showed higher specific bioactivity with the human cells than with the mouse cells. It is likely that subtle differences in the structures of the human and mouse receptors cooperate with small differences in binding affinity to reveal a functionally abnormal mutein. The competition experiments shown in Fig. 5 suggest that the two muteins with the lowest bioactivity (Arg14 → Glu and Arg103 → Ala) fail to prevent binding and activity of wild type Epo. This experiment also rules out any nonspecific suppression by these muteins or the COS cell supernatants of the growth of the Epo-dependent assay cells.

Our structure-function studies suggest that Epo binds to its receptor at the two distinct domains discussed above. Our assignments are consistent with studies on site-directed muteins of other cytokines, including GH, IL-2, IL-3, IL-4, IL-5, and GM-CSF (23), which show that residues on Helix A and Helix D play a critical role in biological activity. X-ray diffraction analysis of the GH-GH receptor complex (13) shows that distinct domains on Helix A and Helix D of GH each bind to a separate receptor molecule, thereby facilitating dimerization of the receptor, perhaps triggering signal transduction.

Acknowledgments—We thank Drs. Fred Cohen, Mark Goldberg, Bob Gruninger, and Linda Mulchay for valuable discussion. We also thank Drs. David Hanks and Jerry Spivak for providing the HCD 57 cell line and Dr. N. Komatsu for the UT7/Epo cell line.

REFERENCES

---

**Fig. 6.** Model of predicted three-dimensional structure of Epo showing a proposed functionally important domain that encompasses Arg14 on Helix A, Arg103 and Ser146 on Helix C, and another that encompasses Glu13, Lys132, and perhaps Asn147, Ser146, Arg143, and Lys146. It is possible that this functional patch also includes a portion of Helix A, analogous to one of the receptor binding domains in GH (13).
Functionally Important Domains of Human Epo