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Site-directed mutants of human myeloperoxidase

A topological approach to the heme-binding site

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Two site-directed mutants of human promyeloperoxidase, MPO(His^{416}→Ala) and MPO(His^{502}→Ala), have been expressed in Chinese hamster ovary cells and purified. Overall purification yields and apparent molecular masses of the mutant proteins were similar to those of the wild-type enzyme. Both mutant species were analyzed spectroscopically to check the presence of the hemic iron in the proteins and were assayed for peroxidasic activity. The data show that substitution of His^{502} leads to the loss, or to an inappropriate configuration, of the heme together with the loss of activity, suggesting that this residue could be the proximal His involved in the binding to the iron centers. On the other hand, substitution of His^{416} by alanine had no effect on either of the studied parameters.

Recombinant myeloperoxidase (human); Site-directed mutagenesis; Heme; Active site

1. INTRODUCTION

Myeloperoxidase (MPO EC 1.11.1.7), a glycosylated hemoprotein present in polymorphonuclear leukocytes, catalyzes the oxidation of halides by hydrogen peroxide to generate potent oxidant species. The mature enzyme is a tetramer composed of two light (10–15 kDa) and two heavy (57–60 kDa) chains [1]. Enzymatic activity depends on the iron atoms, one per heavy chain, in the heme prosthetic group of MPO. The enzyme exhibits a characteristic absorption spectrum with a major red-shifted Soret band, at 428 nm, which is attributed to the unique iron-chlorin prosthetic group. Human recombinant MPO has been recently obtained from engineered Chinese hamster ovary cells. Although it is produced as a glycosylated single chain hemoprotein precursor of 745 amino acid residues (84 kDa), recMPO displays physico-chemical properties very similar to those of the mature natural enzyme [2,3]. The recombinant DNA methodology thus now offers the possibility to identify some amino acid residues involved in the catalytic mechanism and heme binding of MPO using site-directed mutagenesis of the corresponding cDNA, followed by the characterization of expressed mutant proteins. The presence of a proximal histidine ligand to the heme iron in MPO has been indicated by a variety of spectral studies [4,5]. Some evidence has also been presented to support a histidine residue on the distal side of the heme [6,7]. By analogy with the amino acid sequence of thyroid peroxidase, it was proposed that His^{416} of MPO could be the proximal ligand, binding to the iron centers and that His^{502} of MPO could be the distal residue involved in the enzymatic degradation of hydrogen peroxide [8].

The present work consisted of creating site-directed MPO mutants where these two histidine were substituted by alanine residues, and to characterize the modified recombinant proteins in terms of optical absorption spectrum and peroxidasic activity.

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2. MATERIALS AND METHODS

Products used for recombinant DNA experiments, cell culture and protein purification have been listed in a preceding paper [2]. Transfection of recombinant plasmids into CHO cells, selection and culture procedures for transfected cells, protein purification protocols, electrophoretic analysis, Western blotting, ELISA and peroxidase activity assays for recMPO have been detailed previously [2]. The substitution mutants of recMPO, His^{416}→Ala and His^{502}→Ala, were produced by subcloning the entire human MPO coding sequence, excised as a *Hin*dIII–*Sna*bI DNA fragment from plasmid pNIV2703 [2], into M13mp18, which was then used as a template for oligonucleotide-directed mutagenesis using the method of Eckstein [9] (kit from Amersham, UK). After mutagenesis, the appropriate mutated coding sequences were sequenced, then released by restriction digestion and the 423-bp *Apa*I–*Kpn*I fragment comprising the mutations was substituted to its wild-type counterpart in the complete MPO coding cassette carried by the expression vector pNIV2703. Final recombinant plasmids, pNIV2707 and pNIV2708, were transfected into CHO cells. G418-resistant colonies were selected and suspended in six 175 cm^3 Falcon flasks. Cell culture supernatants (1 liter) were collected for each transfected cell line and processed to purify the mutant protein species [2]. Visible spectra were recorded on a Cary 15 spectrophotometer. The presence of the heme in recombinant proteins was assessed by measuring the absorbance at 428 nm. Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380A via the solid-phase phosphoramidite method [10].

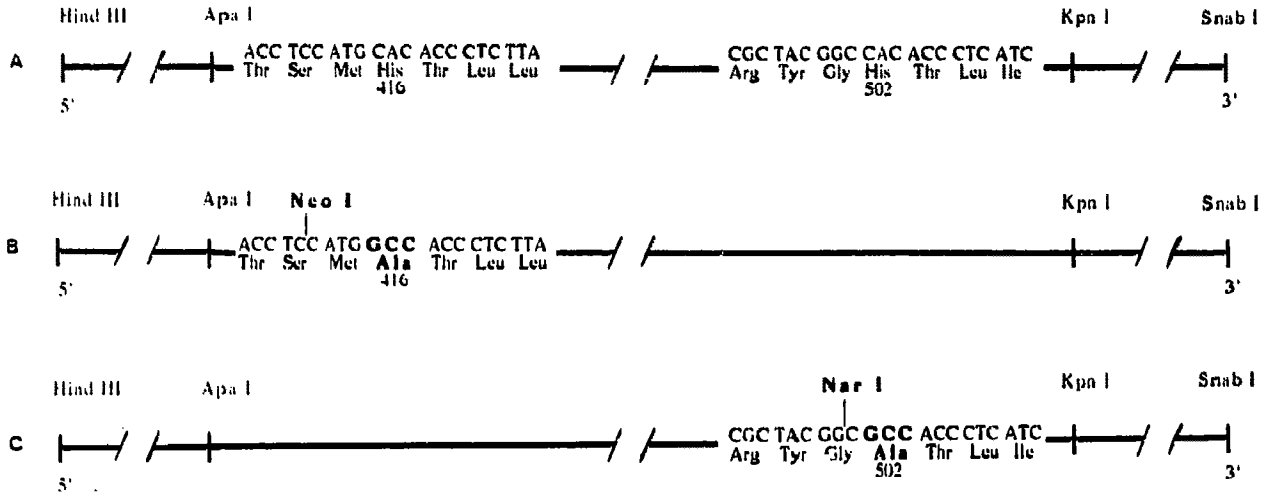

Fig. 1. Schematic representation of the coding cassette for mutant myeloperoxidases. (A) coding cassette for wild-type recMPO in pNIV2703. The *Apa*I–*Kpn*I fragment is represented with nucleotide and amino-acid sequences surrounding His^{416} and His^{502}. (B) coding cassette for His^{416}→Ala recMPO in pNIV2707. (C) coding cassette for His^{502}→Ala recMPO in pNIV2708. In B and C, the 21-mer synthetic oligonucleotides for mutagenesis are shown with the Ala codon and the newly created restriction site in bold. In each case, full lines and -//- indicate the remainder of the *Hin*dIII–*Sna*bI cassette.

3. RESULTS

Two site-directed mutations have been generated into the cDNA coding for human MPO. Both of them substitute putatively important histidine residues, His^{416} and His^{502}, by alanine, a small non-polar amino acid. The mutations were created using the M13mp18 mutagenesis system and oligonucleotides carrying the appropriate mismatches (Fig. 1). After reconstruction of the mutated MPO coding modules, corresponding recombinant plasmids, called pNIV2707 and pNIV2708, respectively, were used to transfect CHO cells in culture. Clones resistant to geneticin, having stably integrated the MPO cDNA, secreted the recombinant proteins in the medium. Two of them, His416-6 and His502-8, producing 1182 and 1435 ng/ml/24 h, respectively, were expanded to obtain 1 liter of culture supernatant, which was used for the purification of the mutant proteins. Overall purification yields for both mutant species were similar to that obtained for the wild-type recMPO (50% final yields). In addition, the purified proteins migrated on SDS-PAGE as single-chain immunoreactive precursors having identical apparent masses as those observed for wild-type recMPO, 84 kDa and 94 kDa [2]. Visible spectra of the purified mutant proteins were compared to that of the wild-type species. As seen in Fig. 2, both wild-type and MPO(His^{416}→Ala) mutant proteins presented a major peak of absorbance at 428 nm (Soret peak) and a minor one at 579 nm. In contrast, the mutant protein MPO(His^{502}→Ala) did not show any visible spectrum at all (Fig. 2).

The specific peroxidasic activity of mutant MPOs was then determined using *o*-dianisidine as substrate [11]. As seen in Fig. 3, MPO(His^{416}→Ala) displayed a similar activity as the wild-type enzyme, whereas the other mutant species, MPO(His^{502}→Ala), was totally inactive in the assay. The specific activity for the wild-type enzyme and MPO(His^{416}→Ala) was 0.118 and 0.106 U/μg, respectively.

4. DISCUSSION

The presence of distal and proximal histidines is a common feature, in many peroxidases, of the heme-binding site (for a recent review see ref. [12]). By analogy with MPO, Kimura et al. suggested that a similar situation should prevail also in human myeloperoxidase and that His^{416} could be the proximal histidine and His^{502},

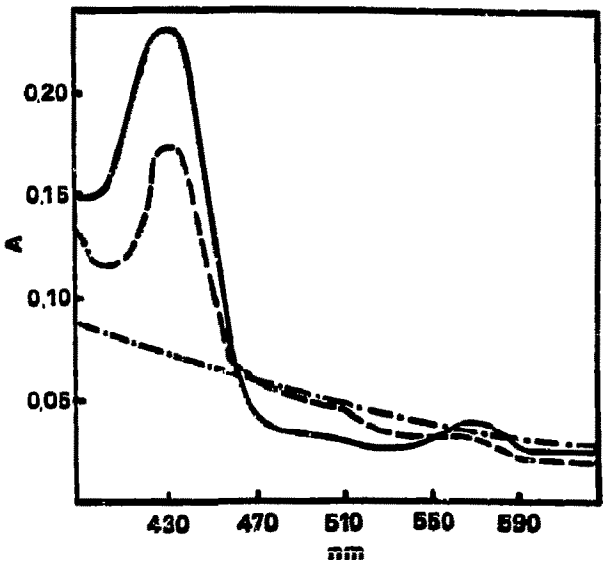

Fig. 2. Visible absorption spectra of recombinant MPOs. (—), wild type recMPO; (- - - -), His^{416}→Ala recMPO; (—·—·), His^{502}→Ala recMPO.

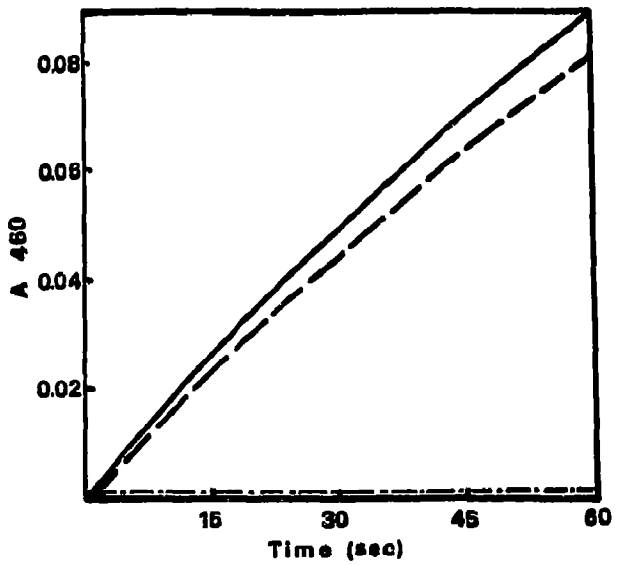

Fig. 3. Peroxidasic activity of recombinant myeloperoxidases using *o*-dianisidine as substrate [10]; the increase in A_{460} was followed for one minute. Identical amounts of proteins (700 ng) were used in each assay. (—), wild type recMPO; (- - - -), His^{416}→Ala recMPO; (–·–·), His^{502}→Ala rec MPO.

or His^{594}, the distal histidine [8]. In this work, we constructed and characterized site-directed mutants of human MPO which carry alanine residues instead of histidine at two of these positions. The results show that His^{502} substituted by alanine led to the loss or inappropriate configuration of the heme together with the loss of peroxidasic activity. We suggest that the His^{502} residue constitutes the proximal histidine, involved in the binding to the iron centers, and not the distal His, as suggested before [8]. On the contrary, mutation of His^{416} had no effect on these parameters, indicating that this His residue does not correspond to the proximal histidine.

The crystal structure of human MPO has currently been elucidated [12]; it will thus be interesting to confront our conclusions to the crystallographic data.

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