CMP-*N*-acetylneuraminic acid hydroxylase: the first cytosolic Rieske iron-sulphur protein to be described in Eukarya

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Abstract Electron paramagnetic resonance (EPR) spectroscopy and analysis of the primary structure of the CMP-*N*-acetylneuraminic acid hydroxylase revealed that this enzyme is the first iron-sulphur protein of the Rieske type to be found in the cytosol of Eukarya. The dithionite-reduced hydroxylase exhibited an EPR signal known to be characteristic for a Rieske iron-sulphur centre (2Fe-2S), the *g*-values being 1.78, 1.91 and 2.01, respectively. An analysis of the primary structure of the hydroxylase led to the identification of an amino acid sequence, known to be characteristic for Rieske proteins. Furthermore, possible binding sites for cytochrome b_5, the substrate CMP-Neu5Ac and a mononuclear iron centre were also identified.

Key words: Hydroxylase; Rieske protein; Sialic acid; Electron paramagnetic resonance; CMP-*N*-acetylneuraminic acid; *N*-Glycolylneuraminic acid

1. Introduction

Sialic acids on the oligosaccharide chains of cell surface glycoconjugates in animals are involved in a number of intercellular recognition and adhesion processes [1,2]. This group of acidic sugars arises mainly from enzymatic derivatisation reactions on *N*-acetylneuraminic acid (Neu5Ac) [2]. The involvement of specific sialic acids in embryogenesis, the immune response, inflammation, tumour cell metastasis and other cellular interactions has aroused considerable interest in the enzymology and regulation of their biosynthesis. One common sialic acid variant, *N*-glycolylneuraminic acid (Neu5Gc), formally results from the hydroxylation of the 5-*N*-acetyl function of Neu5Ac. Glycosylation with Neu5Gc varies considerably with species, tissue and developmental stage [2-4]. In man and chicken, however, Neu5Gc has been only detected in small amounts in various tumour tissues [5-7]. The biosynthesis of Neu5Gc occurs by the action of a hydroxylase specific for CMP-Neu5Ac (EC 1.14.13.45) [8]. This enzyme is of special interest as it seems to play a key role in the regulation of the sialylation of glycoconjugates with Neu5Gc [2].

CMP-Neu5Ac hydroxylase has been purified from mouse liver and pig submandibular gland and found to be a cytosolic monooxygenase, which requires NADH, cytochrome b_5 reductase and cytochrome b_5 for activity and can be stimulated by the addition of iron ions [9-11]. The purified enzyme is monomeric and has a molecular weight of 65 kDa [12-14]. The fact that CMP-Neu5Ac hydroxylase is dependent on cytochrome b_5 and is inhibited by the addition of iron-binding agents [14] suggests that this enzyme may possess an iron-containing prosthetic group. In the following studies we have investigated the nature of this postulated iron cofactor.

2. Materials and methods

2.1. Spectroscopic measurements

The hydroxylase was purified from pig submandibular gland [14] and subjected to EPR spectroscopy in the dithionite-reduced form. The reduction was carried out at room temperature by dissolving a few crystals of sodium dithionite in the enzyme solution. EPR spectroscopy was performed with liquid helium cooling (ESR 910 Cryostat, Oxford Instruments) at 4.2–40.0 K using a Bruker ER 200 EPR spectrometer equipped with an X-band microwave source. The microwave power was 10.0 mW, the modulation amplitude was 10 G and the modulation frequency was 100 kHz. The spectra were quantised with respect to a 1 mM Cu(II) EDTA standard sample measured at 20 K under non-saturating conditions. The *g*-value dependence of intensities was corrected [15]. Additionally, the UV/visible spectrum of the enzyme was determined at room temperature using an Hitachi U-2000 spectrophotometer.

2.2. Cloning of the cDNA of the CMP-Neu5Ac hydroxylase

Purified CMP-Neu5Ac hydroxylase from pig submandibular gland [14] was digested with Lys C and a protein sequence analysis of the resulting peptides was performed using Edman degradation. The sequence information obtained was used to design short (20 bp) degenerate primers for use in polymerase chain reactions with pig submandibular gland cDNA templates. An amplified cDNA fragment was cloned and sequenced. Labeling of this fragment with digoxigenin was performed by polymerase chain reaction with digoxigenin-labeled dUTP. This probe was used to screen an oligo dT primed cDNA library designed from pig submandibular gland mRNA using a cDNA Synthesis Kit (Pharmacia, Heidelberg, Germany), Gigapack II Packaging Extract (Stratagene, La Jolla, CA, USA) and Predigested Lambda Zap II/Eco R I/CIAP Cloning Kit (Stratagene). As the isolated and characterised clones were incomplete at the 5′ region, rapid amplification of cDNA ends (RACE) (5′ Amplifinder RACE Kit, Clontech, Palo Alto, CA, USA) was performed.

3. Results

3.1. Spectroscopic results

The EPR spectroscopic measurements revealed that the dithionite-reduced hydroxylase exhibited a signal known to be characteristic for iron-sulphur proteins of the Rieske type [16], the *g*-values being 1.78, 1.91 and 2.01, respectively (see Fig. 1a). The estimated concentration of enzyme in this measurement was 30 μM and double integration of the signal gave

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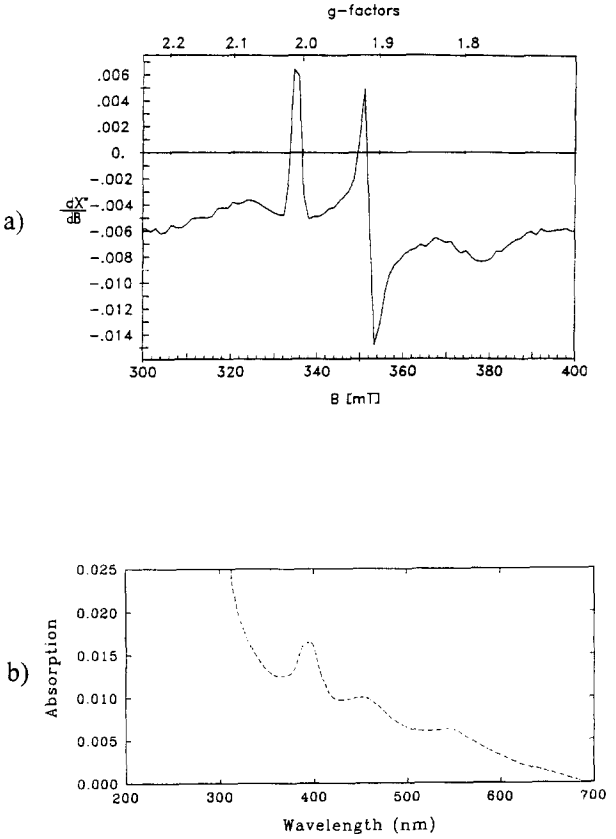

Fig. 1. Spectroscopic characterisation of the pig CMP-Neu5Ac hydroxylase. (a) EPR spectrum of the hydroxylase in the dithionite-reduced form, recorded at 30 K and microwave frequency 9.4284 GHz. (b) UV/visible spectrum of the oxidised enzyme.

a concentration of 12 μM spin, corresponding to 0.4 reduced (2Fe-2S) centres per enzyme monomer. Additionally, the UV/visible spectrum of the oxidized CMP-Neu5Ac hydroxylase exhibited absorbance maxima at about 450 nm and 560 nm which are characteristic of (2Fe-2S) proteins (Fig. 1b).

3.2. cDNA sequencing

Polymerase chain reactions with degenerate hydroxylase-specific oligonucleotide primers led to the amplification of a 96 bp specific cDNA fragment which was cloned and sequenced. A partial open reading frame was found coding for the amino acid sequence expected from peptide sequencing. This cDNA fragment was used to isolate two clones from a cDNA library (see Fig. 2). The cloned cDNAs exhibited the same open reading frame containing all sequence information obtained by amino acid sequencing of the purified hydroxylase, while polyadenylation was observed at different sites in the 3′ non-coding region (see Fig. 2, polyadenylation sites a and b). The 5′ sequence was extended by 5′ RACE and contained an ATG in a KOZAC consensus sequence [16]. However, the molecular weight of the hydroxylase deduced from

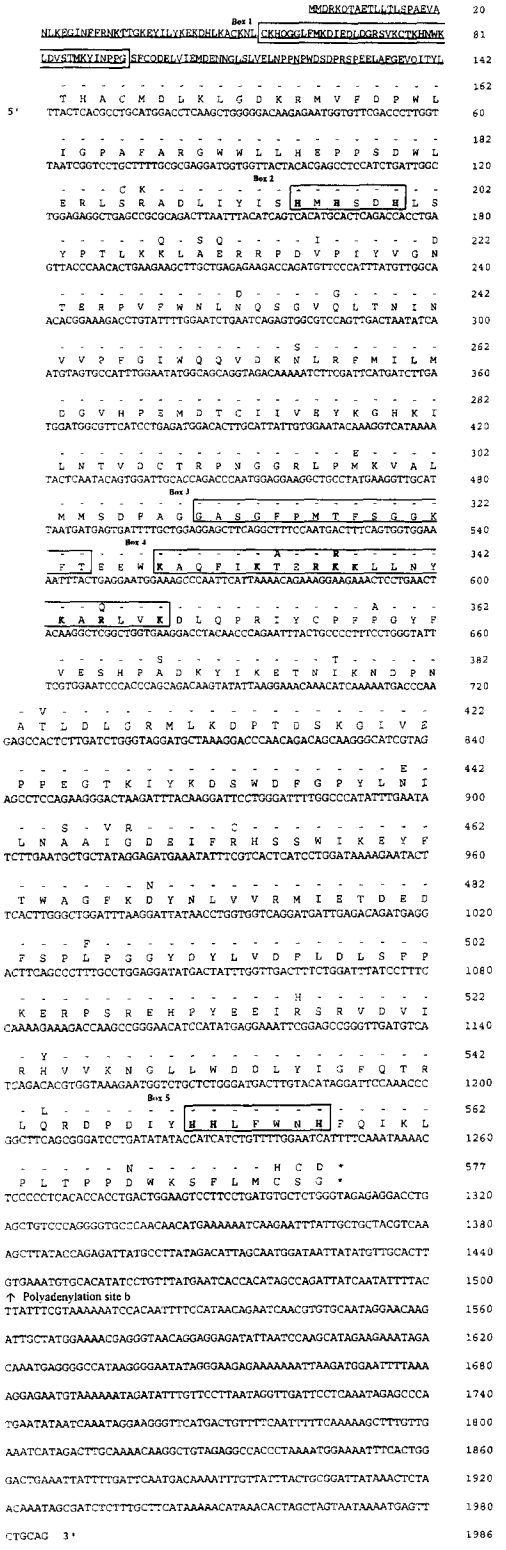

Fig. 2. Primary sequence of CMP-Neu5Ac hydroxylase. The lower line shows the nucleotide sequence of cDNA coding for the pig enzyme and the deduced amino acid sequence is presented directly above. The upper line shows the amino acids which are different in the sequence of the hydroxylase from mouse liver [18]. The N-terminal amino acid sequence of the mouse enzyme is underlined. The amino acid numbering is shown for the mouse hydroxylase sequence. The numbering of the nucleotides relates to the sequence of the pig cDNA described here. The boxes indicate amino acid sequences postulated to be involved in the following functions: box 1, binding of Rieske (2Fe-2S) centre (see also Fig. 3); boxes 2 and 5, binding site for mononuclear iron (critical histidine residues are shown in bold type); box 3, CMP-Neu5Ac binding site (see also Fig. 4); box 4, cytochrome b_5 binding region (the relevant basic residues are shown in bold type). The two polyadenylation sites a and b found in pig submandibular gland cDNA are shown in the 3′ untranslated region of the nucleotide sequence.

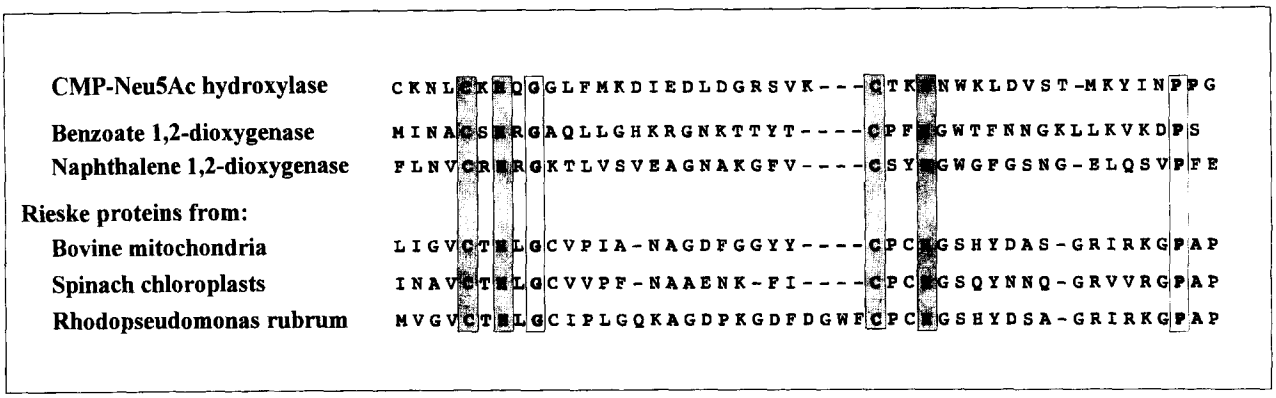

Fig. 3. Possible binding site for a Rieske iron-sulphur centre. The conserved regions of different Rieske-type proteins (benzoate 1,2-dioxygenase and naphthalene 1,2-dioxygenase from *Pseudomonas putida* [20], the Rieske protein in bovine mitochondria, the Rieske protein in the chloroplasts of spinach and the Rieske protein of *Rhodopseudomonas rubrum* [25]) were aligned with the sequence of the CMP-Neu5Ac hydroxylase from the mouse. In all Rieske and Rieske-type proteins, conserved amino acids are boxed. The histidine and cysteine residues considered to be responsible for the binding of the (2Fe-2S) centre are additionally shaded. See also box 1 in Fig. 2.

the amino acid sequence coded by the open reading frame was about 10 kDa less than that obtained by SDS-PAGE of the purified enzyme. An alignment of the amino acid sequences of the hydroxylase from mouse liver, which was published recently [17], and that from pig submandibular gland suggests that the sequence of the pig enzyme is not complete at the 5′ region (see Fig. 2). The existence of different forms of mRNA for the CMP-Neu5Ac hydroxylase is possible and is under investigation. Nevertheless, the aligned sequences were 92% identical.

4. Discussion

These spectroscopic investigations on the nature of the iron cofactor in CMP-Neu5Ac hydroxylase strongly suggest the presence of a Rieske-type (2Fe-2S) cluster [16]. The content of Rieske centres (0.4 per enzyme monomer) may result from their loss during the rather lengthy purification of the enzyme. Rieske iron-sulphur proteins have so far only been described in Prokarya and Archaea as well as in organelles proposed to originate from bacterial symbionts, i.e. mitochondria and chloroplasts. The discovery of a Rieske centre in a cytosolic enzyme from Eukarya is therefore a novel observation. To support this conclusion, the primary structure of the hydroxylase was investigated. An inspection of the complete sequence of the hydroxylase from mouse liver [18] revealed a region conserved in Rieske iron-sulphur proteins (see Fig. 3) consisting of two boxes each with a cysteine and a histidine residue separated by about 20 amino acids near the N-terminus [16,19-22]. The importance of these residues for the binding of the (2Fe-2S) group has been shown for some Rieske proteins by site-directed mutagenesis [23-25]. The relevance of the conserved glycine and proline residues (see Fig. 3) is not yet clear. Although the N-terminal primary structure of the pig enzyme could not be determined by cDNA sequencing, the 92% identity of the obtained sequence with the analogous region of the mouse enzyme suggests that the remaining 5′ sequence of the pig enzyme is also homologous with the mouse hydroxylase. Indeed, the identical molecular weight and catalytic properties of both enzymes [12-14] underscore their structural similarity.

Since (2Fe-2S) groups do not usually incorporate exogenous iron in vitro, the activatory effect of Fe^{2+} ions on the hydroxylase [13,14] may be due to the formation of a mononuclear iron centre. A combination of a Rieske (2Fe-2S) centre and mononuclear iron was proposed to exist in the benzoate and phthalate dioxygenases of bacterial origin [19,26] and also in the O_2-activating component of 4-methoxybenzoate monooxygenase from *Pseudomonas putida* [27]. Several investigations indicate that oxygen binding and activation occur at the mononuclear iron centre [16]. Such an iron centre might bind to histidine clusters [16] which are present in the sequence of the hydroxylase (see Fig. 2). Neidle et al. [19] suggested that the sequence $YHX_{4-5}H$, which is highly conserved in bacterial Rieske dioxygenases, may be responsible for the binding of a mononuclear iron centre. This sequence is very similar to the hydroxylase histidine clusters (see Fig. 2).

A search through the EMBL Sequence Database with the cDNA of the porcine hydroxylase revealed no obvious sequence homologies to other proteins. We therefore searched for further shorter homologous regions, taking into account the catalytic properties of the hydroxylase.

In addition to the CMP-Neu5Ac hydroxylase, several other proteins interact with cytochrome b_5. An electrostatic interaction between a region rich in basic residues on cytochrome P450 enzymes and an acidic sequence motif of cytochrome b_5 was discussed by Davydov et al. [28]. A similar sequence with several lysine and arginine residues lying close together was found in the primary structure of the hydroxylase (see Fig. 2).

In order to localize a potential CMP-Neu5Ac binding site,

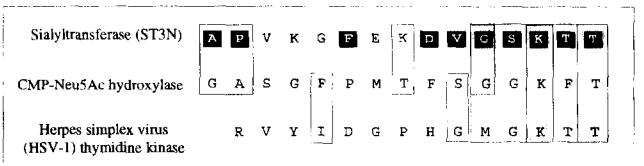

Fig. 4. Possible binding site for the substrate CMP-Neu5Ac. A highly conserved region of the sialyltransferases, the so-called sialyl motif, and the thymidine binding region of the thymidine kinase from herpes viruses are compared with the sequence of the CMP-Neu5Ac hydroxylase (see also Fig. 2, box 3). Amino acids that are identical are shaded, amino acids that are either identical or whose coding triplets differ in one position are in a box. Amino acids that are identical in the sialyltransferases: Galβ1,3(4)GlcNAc α2,3-sialyltransferase (ST3N), Galβ1,3GalNAc α2,3-sialyltransferase (ST3O) and Galβ1,4-GlcNAc α2,6-sialyltransferase (ST6N) [32] are printed white on black.

we compared a highly conserved region of the sialyltransferases, the so-called sialyl motif [29], with the sequence of the hydroxylase and found a similar region (see Fig. 4). This region of the sialyltransferases has recently been shown to be part of the binding site for CMP-sialic acids by site-directed mutagenesis [30]. A sequence of five amino acids which were similar or identical to this region was also found in the nucleotide-binding region of the thymidine kinase from herpes viruses [31]. The structural similarity of thymidine and cytidine supports the hypothesis that the presented sequence of the hydroxylase is involved in pyridine nucleotide binding (see Fig. 4).

Of all these possible binding sites, the existence of a Rieske iron-sulphur centre in the CMP-Neu5Ac hydroxylase is of particular interest, as it poses new questions about the evolution of these iron-sulphur proteins. Since Rieske proteins had previously only been described in prokarya and archaea as well as in organelles proposed to originate from bacterial symbionts, they were considered to be phylogenetically very old [16]. In contrast to this, the CMP-Neu5Ac hydroxylase probably appeared late in evolution, since the echinoderms are the most primitive organisms known to produce Neu5Gc [2]. The independent development of a Rieske iron-sulphur centre in this hydroxylase cannot be excluded. However, a shuffling of the conserved Rieske (2Fe-2S) binding motif also seems possible, since the Rieske iron-sulphur protein of mitochondria is coded by the nuclear DNA [25].

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