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Purification, characterization and reconstitution of CMP-*N*-acetylneuraminate hydroxylase from mouse liver*

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CMP-*N*-acetylneuraminate hydroxylase was isolated from mouse liver high speed supernatant with a yield of 0.4% and an apparent 1000-fold purification. The enzyme is a monomeric protein with a molecular weight of 66 kDa, as determined by gel filtration and SDS-PAGE. The hydroxylase system was reconstituted with Triton X-100-solubilized mouse liver microsomes and purified soluble or microsomal forms of cytochrome b_5 reductase and cytochrome b_5. The systems were characterized in detail and kinetic parameters for each system were determined.

Keywords: sialic acid; N-glycoloylneuraminic acid; hydroxylase; protein purification; cytochrome b_5; electron transfer; enzyme system reconstitution

Abbreviations: Neu5Ac, *N*-acetyl-β-D-neuraminic acid; Neu5Gc, *N*-glycoloyl-β-D-neuraminic acid; CMP-Neu5Ac, cytidine-5′-monophospho-*N*-acetylneuraminic acid; CMP-Neu5Gc, cytidine-5′-monophospho-*N*-glycoloylneuraminic acid; TCA, trichloroacetic acid; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; SOD, superoxide dismutase.

Enzymes: CMP-*N*-acetylneuraminate: NADH oxidoreductase (N-acetyl hydroxylating) (E.C. 1.14.13.45), CMP-Neu5Ac hydroxylase; NADH: cytochrome b_5 oxidoreductase (E.C. 1.6.2.2), cytochrome b_5 reductase; hydrogen peroxide: hydrogen peroxide oxidoreductase, catalase (E.C. 1.11.1.6); superoxide:superoxide oxidoreductase (E.C. 1.15.1.1), superoxide dismutase.

Introduction

Sialic acid is a general name for a group of about 30 structurally related acidic sugars [1], which are present in the oligosaccharide chains of many glycoconjugates of animal and microbial origin. They have been shown to be involved in a number of cellular recognition and adhesion processes [2, 3]. The structural heterogeneity of the sialic acids is due to the variety of biosynthetic modifications that *N*-acetylneuraminic acid (Neu5Ac), the simplest and most ubiquitous sialic acid, can undergo. The most common derivative of Neu5Ac is *N*-glycoloylneuraminic acid (Neu5Gc), which formally results from a hydroxylation of the 5-*N*-acetyl methyl function of Neu5Ac.

Neu5Gc occurs in most animal groups throughout the deuterostomate lineage [4], the extent of glycosylation with Neu5Gc varying considerably with species, tissue and developmental stage. In normal human and chicken tissues Neu5Gc is absent, but it has been detected in small amounts in cancerous tissues [5, 6], thus making the clarification of the mechanisms involved in governing the expression of this sialic acid particularly interesting.

The hydroxylation of Neu5Ac occurs on the level of its CMP-glycoside and is catalysed by the action of CMP-*N*-acetylneuraminate hydroxylase, giving CMP-Neu5Gc as the immediate product [7, 8]. CMP-Neu5Gc is utilized by the glycosylation machinery of the Golgi apparatus in the same way as CMP-Neu5Ac [9], suggesting that the rate of CMP-Neu5Gc production by CMP-Neu5Ac hydroxylase is important in regulating the extent of sialylation with Neu5Gc. The significance of the hydroxylase in controlling Neu5Gc incorporation has been shown in several studies [9–13].

CMP-Neu5Ac hydroxylase has been studied intensively in fractionated homogenates of several tissues, including pig submandibular glands [7], mouse liver [8, 14–16] and starfish gonads [17] and has been found to be a soluble, probably cytosolic monooxygenase that is dependent on NADH. This enzyme can be activated by exogenously added iron salts and is inhibited by several iron-binding compounds, possibly implicating the involvement of a non-haem iron cofactor. Fractionation experiments performed to purify CMP-Neu5Ac hydroxylase from mouse

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* This paper is dedicated to Professor Harry Schachter on the occasion of his 60th birthday.

liver revealed that this enzyme is dependent on two other proteins which have been identified as cytochrome b_5 [14–16] and cytochrome b_5 reductase [13, 18, 19], thus forming an electron transport chain in which the monooxygenase is the terminal electron acceptor. A similar mechanism is also proposed for the hydroxylase from pig submandibular glands [20], suggesting that this is a common hydroxylase system, at least among mammals.

Cytochrome b_5 reductase and cytochrome b_5 are well characterized electron carriers delivering reducing equivalents from NADH to several enzymes of cellular metabolism [21], including many monooxygenases. Both proteins exist in amphiphilic and soluble forms. The amphiphilic forms consist of a hydrophobic membrane anchoring domain and a hydrophilic catalytic domain, which can be separated by proteolytic cleavage [22, 23]. They are mainly located in the membranes of the endoplasmatic reticulum, with their catalytic domains directed towards the cytoplasm [24]. The soluble forms differ from the amphiphilic forms chiefly by the absence of the membrane anchor and are mainly found in the cytosol of erythrocytes, where they function in the reduction of adventitiously oxidized haemoglobin [25].

Since in mouse liver high speed supernatant, both forms of cytochrome b_5 reductase and cytochrome b_5 could be identified [19], it is of interest to characterize their interaction with the hydroxylase. Reconstitution experiments performed using crude CMP-Neu5Ac hydroxylase fractions with soluble and amphiphilic forms of cytochrome b_5 revealed that the hydroxylase accepts electrons from both forms of this electron carrier, the soluble form, however, is preferred [18, 19]. Since influences of other cellular factors cannot be excluded, these reconstitution experiments have to be performed under defined conditions, with all three purified protein components. Recently, the purification of the terminal monooxygenase from mouse liver has been reported [26], but no reconstitution in a purified system has been described.

In this work we describe a purification procedure for the CMP-Neu5Ac hydroxylase as well as reconstitution experiments with Triton X-100-solubilized mouse liver microsomes as well as purified soluble and amphiphilic cytochrome b_5 reductase and cytochrome b_5. The conditions for reconstitution were determined and each system was characterized. A part of this work has been published in preliminary form [27].

Materials and methods

Materials

Analytical grade reagents from Sigma Chemicals Ltd (Deisenhofen, FRG), Merck (Darmstadt, FRG) and Boehringer (Mannheim, FRG) were used throughout this study. CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac (250–300 mCi mmol^{-1}) was obtained from Amersham (Braunschweig, FRG). The chromatographic materials were purchased from the following companies: Superose S.12, NAP-5, NAP-10 and PD-10 columns as well as Q-Sepharose from Pharmacia (Freiburg, FRG), Cibacron Blue 3GA-Agarose (type 3000-CL-L), Reactive Brown 10-Agarose and 5′-ADP-Agarose from Sigma Chemicals Ltd (Deisenhofen, FRG). Trypsinized cytochrome b_5 covalently attached on CH-Sepharose was a gift of Jens Carlsen and Kirsten Christiansen, Panum Institute, University of Copenhagen.

Assay for CMP-Neu5Ac hydroxylase

In standard assays, CMP-Neu5Ac hydroxylase activity was measured in 50 mM Hepes/NaOH, pH 7.4, at 37 °C in the presence of Triton X-100 (0.5% by weight final concentration) solubilized mouse liver microsomes (about 60 µg protein per assay), 10 µM CMP-[^{14}C]Neu5Ac, 1 mM NADH and 0.5 mM $FeSO_4$ in a final volume of 30 µl. Reactions were stopped by the addition of 5 µl 1 M trichloroacetic acid (TCA), the precipitated material removed by centrifugation and the released [^{14}C]sialic acids analysed using radio thin-layer chromatography, as previously described [8]. All hydroxylase assays described were performed in duplicate.

For reconstitution experiments, mouse liver microsomes were replaced by the purified amphiphilic and soluble forms of cytochrome b_5 reductase and cytochrome b_5, in amounts depending on the experiment performed, as indicated in the Results section. In assays containing the soluble forms of these proteins, no detergent was added.

In time course experiments, the assay volumes were doubled, and after selected time intervals, 10 µl aliquots of each assay were removed, added to 5 µl TCA and analysed as above.

In several reconstitution experiments, Triton X-100 was replaced with either Chaps or octylglycoside (both from Sigma Biochemicals Ltd, Deisenhofen, FRG), at optimal concentrations of 10 and 30 mM, respectively, as given in [15]. In some assays, 3000 Units of bovine liver catalase (Sigma Biochemicals Ltd, Deisenhofen, FRG) or of bovine erythrocyte superoxide dismutase (Boehringer, Mannheim, FRG) were added. Mannitol and glutathione were added to a final concentration of 1 mM. All added enzymes or compounds were dissolved in 50 mM Hepes/NaOH, pH 7.4.

Assays for cytochrome b_5 reductase

Ferricyanide and cytochrome c reducing activities of mouse liver microsomes as well as soluble and amphiphilic cytochrome b_5 reductase were determined using modifications of methods already described [27, 28]. To simulate the conditions used in the hydroxylase assays, the activities of microsome bound and purified amphiphilic reductases were assayed in presence of 0.5% Triton X-100 (by weight), while soluble reductase was assayed without detergent.

The ferricyanide assays were performed in duplicate at 37 °C in 50 mM Hepes/NaOH, pH 7.4, in the presence of 0.5 mM potassium ferricyanide and 0.25 mM NADH in a total volume of 1 ml. The reduction of ferricyanide was

started by addition of enzyme and followed photometrically at 420 nm. The reduction rates were calculated using an extinction coefficient of 1.02 mM^{-1} cm^{-1} for ferricyanide.

The assays for cytochrome c reductase were performed in duplicate at 37 °C in 50 mM Hepes/NaOH in the presence of 0.5 mg ml^{-1} horse heart cytochrome c (type III, Sigma Biochemicals Ltd, Deisenhofen) and 1 mM NADH, in a final volume of 0.6 ml. When the purified amphiphilic and soluble reductases were tested, 32 pmol of the respective form of cytochrome b_5 was added. No additional cytochrome b_5 was used in experiments with microsomes. The reactions were started by addition of reductase and the reduction of cytochrome c was followed photometrically at 550 nm. Reduction rates were determined using an extinction coefficient of 18.7 mM^{-1} cm^{-1} [30].

Preparation of microsomes and high speed supernatant from mouse liver

Freshly excised livers from 70 male Balb/c mice were homogenized with eight strokes of a Potter-Elvehjem homogenizer in nine volumes (ml per g wet tissue) of 50 mMHepes/NaOH, pH 7.4, containing 0.25 M sucrose. To remove larger cell fragments, the homogenate was centrifuged at 10000 × **g** for 20 min and microsomes were collected by centrifugation of the supernatant at 100000 × **g** for 60 min. The resulting supernatant was used for purification of the hydroxylase. The microsomal pellet was resuspended in the previous volume of 50 mM Hepes/NaOH, pH 7.4, and the latter centrifugation step was repeated. The washed microsomes were resuspended in the latter buffer (10 ml g^{-1} starting tissue), giving a protein concentration of about 16 mg ml^{-1}, aliquotted and stored at −20 °C until use.

Purification of CMP-Neu5Ac hydroxylase

All purification steps were performed at 4 °C. Unless stated otherwise, hydroxylase fractions containing NaCl were desalted using prepacked Sephadex G-25 columns (NAP-5, NAP-10 or PD-10) equilibrated in 50 mM Hepes/NaOH, pH 7.4, before determining the activity using the standard assay with solubilized microsomes. Protein samples were concentrated by ultrafiltration on membranes with a M_r cut-off of 10000 Da (Sartorius, Göttingen, FRG). Protein concentrations were determined using the Bio-Rad reagent (Bio-Rad, Munich, FRG) with bovine serum albumin as standard.

Fractionation on Q-Sepharose. Freshly prepared high-speed supernatant was applied to a 4.5 × 19 cm column of Q-Sepharose equilibrated in 10 mM Hepes/NaOH, pH 7.4, with a flow rate of 5 ml min^{-1}. Non-bound material was removed with two column volumes of equilibration buffer and the column was developed with a linear gradient of 0–0.6 M NaCl in the same buffer in a total volume of 4.4 l, at a flow rate of 3 ml min^{-1}. Fractions exhibiting more than 30% of the maximal hydroxylase activity were pooled, concentrated and desalted, giving a final volume of 13 ml.

Fractionation on Cibacron Blue 3GA-Agarose. The hydroxylase pool obtained from Q-Sepharose was applied to a 3.1 × 15.2 cm column of Cibacron Blue 3GA-Agarose equilibrated with 50 mM Hepes/NaOH, pH 7.4. The flow was stopped for about 30 min to allow optimal binding, after which the column was washed with about three column volumes of equilibration buffer. Elution was performed with a linear gradient of 0–0.6 M NaCl in 50 mM Hepes/NaOH, pH 7.4, in a total volume of 1.4 l, at a flow rate of 1.4 ml min^{-1}. Fractions containing more than 30% of the maximal hydroxylase activity were pooled, concentrated and desalted in a final volume of 3.5 ml.

Fractionation on Reactive Brown 10-Agarose. The hydroxylase fraction from Cibacron Blue 3GA-Agarose was applied to a 0.75 × 5 cm column of Reactive Brown 10-Agarose, equilibrated in 50 mM Hepes/NaOH, pH 7.4. The flow was stopped for 20 min and unbound material was removed with about three column volumes of equilibration buffer. The column was developed with a 120 ml linear gradient of 0–1 M NaCl in the latter buffer, performed at a flow rate of 0.12 ml min^{-1}. Fractions containing more than 30% of the maximal hydroxylase activity were pooled, equilibrated in the starting buffer by repeated dilution and ultrafiltration, and finally resuspended in 300 µl of 50 mM Hepes/NaOH, pH 7.4, containing 0.1 M NaCl.

Fractionation on Superose S.12. Gel filtration on a Superose S.12 column, equilibrated in 50 mM Hepes/NaOH, pH 7.4, containing 0.1 M NaCl, was performed as a final purification step. The hydroxylase pool obtained from Reactive Brown 10-Agarose was fractionated in two separate runs, eluting with a flow rate of 0.2 ml min^{-1}. Fractions of 0.2 ml were collected and assayed for hydroxylase activity without prior desalting. Active fractions were also analysed using SDS-PAGE. The purest fractions exhibiting the highest hydroxylase activity were pooled. These pools from both runs were combined, equilibrated in 50 mM Hepes/NaOH, pH 7.4, by repeated dilution and ultrafiltration and concentrated to a final volume of 260 µl.

Determination of the molecular weight using SDS-PAGE and gel filtration

SDS-polyacrylamide gel electrophoresis was performed, with a 4% acrylamide stacking gel and a 10% acrylamide resolving gel [31]. Protein bands were visualized using silver staining [32]. The following proteins were used as molecular weight markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 kDa)

The native molecular weight of the hydroxylase was

determined from the elution volume obtained from the last purification step, i.e. gel filtration on Superose S.12. Gel filtration of marker proteins was performed under the same conditions as in the purification of the hydroxylase. The proteins used were as follows: cytochrome c (12.5 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and bovine serum albumin (68 kDa).

Preparation of amphiphilic and soluble forms of cytochrome b_5 reductase and cytochrome b_5 from fractionated mouse liver

Preparation of amphiphilic forms of cytochrome b_5 reductase and cytochrome b_5. These two proteins were purified from mouse liver microsomes as described in [19].

Preparation of soluble cytochrome b_5 reductase. High speed supernatant was fractionated on Q-Sepharose as described for the hydroxylase. Fractions containing ferricyanide reducing activity were pooled, concentrated and equilibrated in 50 mM Hepes/NaOH, pH 7.4, containing 0.25 mM EDTA. This pool was applied to a 0.75 × 2.8 cm column of 5′-ADP-Agarose equilibrated in the latter buffer. After washing with three column volumes, reductase was eluted using 0.3 mM NADH in the same buffer. The eluate was concentrated and equilibrated in 10 mM Hepes/NaOH, pH 7.4, and applied on to a 1.5 ml column of trypsinized cytochrome b_5 covalently attached to CH-Sepharose, equilibrated in the same buffer. After washing the column to remove unbound protein, elution was performed with 1 M NaCl in the equilibration buffer. The eluate was concentrated by ultrafiltration and equilibrated in 50 mM Hepes/NaOH, pH 7.4 in a final volume of 450 µl. This preparation exhibited one main protein band on SDS-PAGE of 30 kDa. The method described gave a yield of about 5% reductase activity and a 240-fold enrichment.

Preparation of soluble cytochrome b_5. Two ml of a 4.1 µM solution of purified amphiphilic cytochrome b_5 was digested with 6 µg of bovine pancreatic trypsin (Sigma Chemicals Ltd, Deisenhofen) for 41 h at 4 °C, followed by the addition of a stoichiometric amount of bovine lung aprotinin (Sigma Chemicals Ltd, Deisenhofen). SDS-PAGE revealed complete digestion of the amphiphilic form to the soluble form of cytochrome b_5.

Results and discussion

Purification of CMP-N-acetylneuraminate hydroxylase

CMP-Neu5Ac hydroxylase was purified from mouse liver high-speed supernatants using the procedure described in Materials and methods. The elution profiles obtained from the various column chromatographic steps are shown in Fig. 1 and the respective protein patterns obtained by SDS-PAGE are shown in Fig. 2. A quantitative assessment of the purification procedure is presented in Table 1.

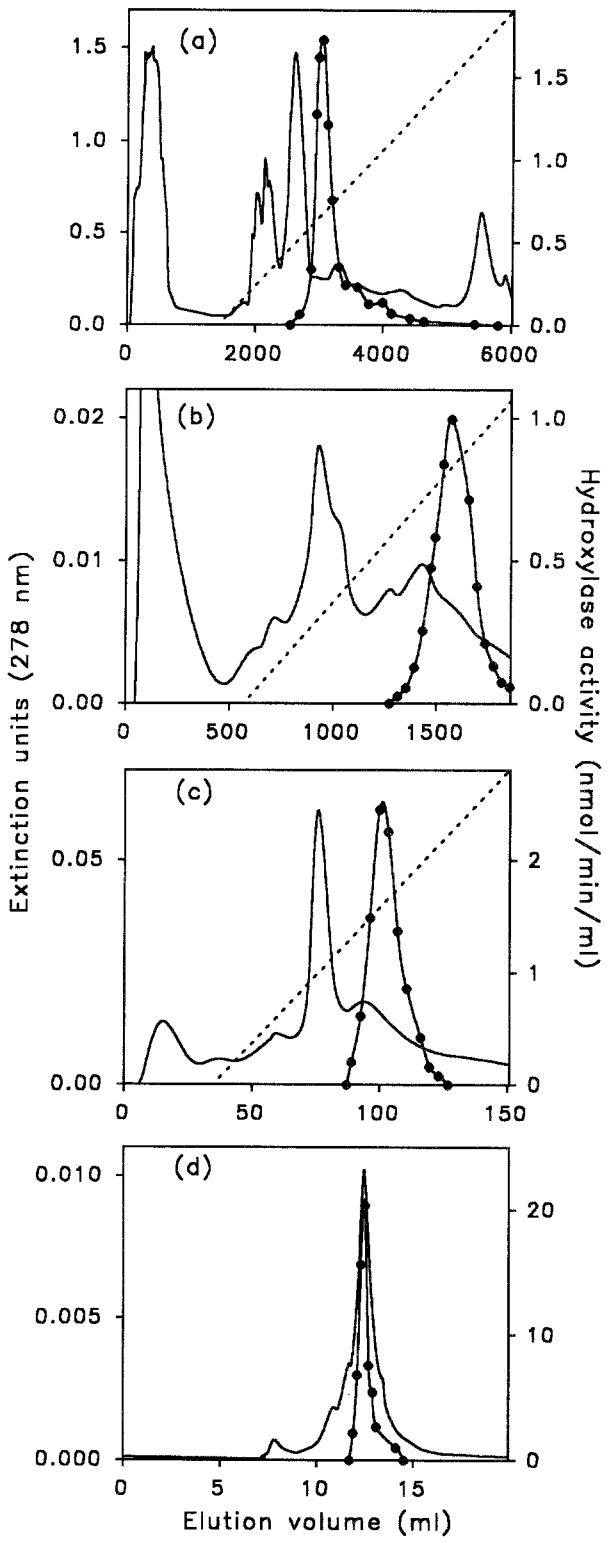

Figure 1. Elution profiles of the different chromatographic steps in CMP-Neu5Ac hydroxylase purification. (a) Q-Sepharose, (b) Cibacron Blue 3GA-Agarose, (c) Reactive Brown 10-Agarose and (d) Superose S.12. Solid lines show the protein profile, dots indicate hydroxylase activities determined using the standard assay and the dashed lines show the course of the gradients: (a) 0–0.6 M; (b) 0–0.52 M; (c) 0–1 M NaCl.

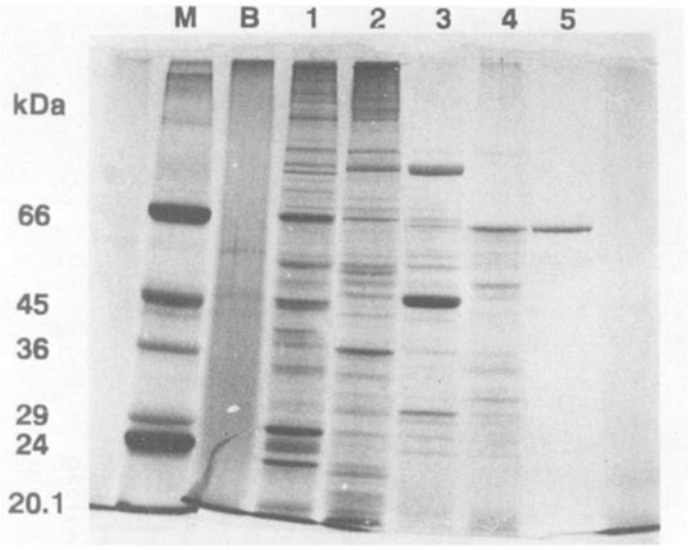

Figure 2. Silver stained SDS-PAGE of the various stages during the purification of CMP-Neu5Ac hydroxylase. Samples: M. Marker proteins; B. Buffer (50 mM Hepes/NaOH, pH 7.4); 1. 100 000 × **g** supernatant (5 μg protein); 2. Q-Sepharose eluate (4 μg); 3. Cibacron Blue 3GA-Agarose eluate (4 μg); 4. Reactive Brown 10-Agarose eluate (3 μg); 5. Superose S.12 (0.8 μg).

Table 1. Summary of purification data for CMP-Neu5Ac hydroxylase.

Fraction	*Specific enzyme activity (nmol min^{-1} per mg protein)*	*Total enzyme activity (nmol min^{-1})*	*Purification factor*	*Yield (%)*
100 000 × **g** supernatant	0.13	851	1	100
Q-Sepharose	2.3	308	18	36
Cibacron Blue 3GA-Agarose	17.4	119	134	14
Reactive Brown 10-Agarose	64.9	29	499	3.4
Superose S.12	126	3.3	969	0.4

Since hydroxylase activity could only be determined when cytochrome b_5 reductase and cytochrome b_5 are present, both proteins had to be added to the fractions when assayed. The use of Triton X-100-solubilized mouse liver microsomes as a source for these components, as proposed in [19], was a helpful aid in assaying the hydroxylase.

The rather shallow NaCl gradients used during chromatography on Q-Sepharose, Cibacron Blue 3GA-Agarose and Reactive Brown 10-Agarose (13–15 column volumes) were found to be necessary to ensure a good separation of the hydroxylase from contaminating proteins. Fractionation on Reactive Brown 10-Agarose was found to be especially important for the purification, resulting in the hydroxylase being the main component of the protein mixture obtained (Fig. 2). The residual contaminating proteins were finally eliminated by gel filtration on Superose S.12.

The final yield of activity is relatively low (0.4%). However, storage of a crude high-speed supernatant for 9 days at 0 °C only led to a 16% decrease in hydroxylase activity, suggesting that the enzyme is intrinsically stable. Thus the observed low recovery is probably a result of inactivation or losses due to the small amounts of protein being handled in the final steps of the purification. From 100 g starting tissue (livers from about 70 mice) 26 μg of purified hydroxylase was obtained. Mouse liver thus does not seem to be an optimal source for the large amounts of protein necessary for detailed structural investigations or for antibody production. Recent investigations suggest that pig submandibular glands are a more convenient source of CMP-Neu5Ac hydroxylase [33].

The purified hydroxylase gave rise to a single protein band on SDS-PAGE with an apparent molecular weight of 66 kDa (Fig. 2). The native molecular weight of the hydroxylase was determined by gel filtration on a calibrated Superose S.12 column to be 58 kDa (Fig. 3), indicating that this enzyme is a monomer. These data are consistent with those determined previously for mouse liver hydroxylase [19, 26] and for the CMP-Neu5Ac hydroxylase isolated from pig submandibular glands [33], suggesting that this is a common property of CMP-Neu5Ac hydroxylase from mammalian tissues.

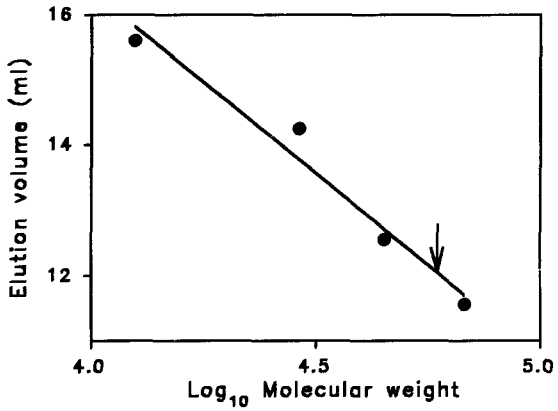

Figure 3. Determination of molecular weight of the native CMP-Neu5Ac hydroxylase. Elution volumes of the marker proteins (●) and of CMP-Neu5Ac hydroxylase (arrow) from Superose S.12 were determined. The molecular weight of the hydroxylase was calculated by linear regression.

Reconstitution of the purified CMP-Neu5Ac hydroxylase system

Purified CMP-Neu5Ac hydroxylase was assayed together with either purified soluble cytochrome b_5 reductase and cytochrome b_5 (soluble system) or the purified amphiphilic, microsome-derived forms of these two proteins (amphiphilic system). For comparison, the hydroxylase was also assayed

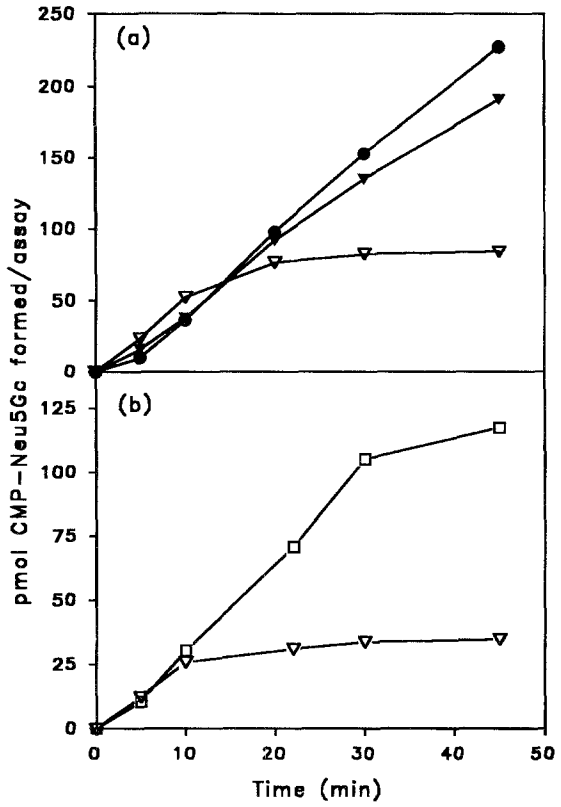

Figure 4. Reconstitution of the complete CMP-Neu5Ac hydroxylase system. (a) The time course of CMP-Neu5Gc production was determined using purified hydroxylase and purified soluble (—▼—) or amphiphilic (—▽—) cytochrome b_5 and cytochrome b_5 reductase or mouse liver microsomes (—●—). Ferricyanide reductase activities were 3.6 μmol min^{-1} in each assay, and the concentration of cytochrome b_5 in each purified system was 5 μM. (b) The time course of CMP-Neu5Gc production in the system reconstituted with amphiphilic cytochrome b_5 and cytochrome b_5 reductase under similar conditions as in (a) in the absence (—▽—) and presence (—□—) of catalase (3000 U).

with Triton X-100-solubilized mouse liver microsomes. The time course of CMP-Neu5Ac hydroxylation in all three systems is shown in Fig. 4a.

In the standard system based on solubilized microsomes, a linear hydroxylation rate was maintained over 40 min, until most of the substrate was consumed. The soluble system exhibited similar behaviour, whereas with the amphiphilic system, a linear reaction rate was maintained only over 10–15 min, followed by rapid inactivation. This result was unexpected, since the hydroxylase in both purified systems was assayed under comparable conditions. The main difference between the two systems is the presence of Triton X-100 in the assays performed with the amphiphilic system. This detergent was added since it improves the accessibility of amphiphilic cytochrome b_5, as described previously [18, 19]. Triton X-100, however, is known to form peroxides during autoxidation [34], which may damage the enzyme system. Assaying the amphiphilic system with

Table 2. Effect of detergents used in reconstitution experiments performed with the amphiphilic system on the activity of the hydroxylase.

Detergent	*Initial rate*[a] (%)	*Effect on linearity*
Triton X-100	100	–
Chaps	76	–
Octylglycoside	96	–
None	55	–

[a] Values are given in relation to those determined with the amphiphilic system using Triton X-100.

Table 3. Modifications of the amphiphilic system performed to identify the reactive oxygen species responsible for hydroxylase inactivation.

Modification	*Initial rate*[a] (%)	*Effect on linearity*
Catalase (3000 U)	130	+
H_2O_2 (1 mM)	0	–
Mannitol (1 mM)	100	–
Glutathione (1 mM)	84	–
Superoxide dismutase (3000 U)	50	–

[a] Values are given in relation to the non-modified system.

Chaps or octylglycoside instead of Triton X-100 or in the absence of detergent resulted in different initial rates; the time-dependent behaviour, however, was similar (Table 2).

Oxygenase reactions as well as electron transfer mechanisms may produce reactive oxygen species. This may lead to the consumption of molecular oxygen, a proposed substrate for the hydroxylase reaction, which may reduce its concentration to a limiting level, or to a poisoning of the enzyme system. The former possibility can be excluded, since addition of air-equilibrated buffer after inhibition of the system did not lead to a restarting of CMP-Neu5Ac hydroxylation. Experiments performed to clarify the possible participation of reactive oxygen in the inactivation of the amphiphilic hydroxylase system are summarized in Table 3.

Only the addition of catalase led to a linearization of the hydroxylation time course in the amphiphilic system, as shown in Fig. 4b. Moreover, this enzyme gave rise to an increase in the initial rate. Since the addition of hydrogen peroxide is inhibitory, this points to its involvement in the observed inactivation. The addition of mannitol did not exhibit any effect on the behaviour of the amphiphilic system, suggesting that hydroxyl radicals, possibly derived from interaction of hydrogen peroxide with Fe(II)-ions in the Fenton reaction [35], were not involved. Interestingly, the addition of superoxide dismutase led to a significant decrease of the initial hydroxylation rate, possibly due to

the accumulation of hydrogen peroxide, the product of superoxide radical dismutation. Thus, the existence of superoxide radicals in the assay is likely. Furthermore, the hydrogen peroxide presumed to be formed in the absence of SOD may be derived from superoxide radicals, since the dismutation also takes place spontaneously [36]. From these results, the formation of hydrogen peroxide and of superoxide radicals are both likely to occur during incubation with the amphiphilic system. The inactivation of this system may, however, primarily be due to the formation of hydrogen peroxide. Possible sources of these reactive oxygen species are discussed in the following.

In all three reconstituted systems, the hydroxylase fraction was identical. It is therefore unlikely that this enzyme is responsible for the inhibition observed in the amphiphilic system. A more likely candidate is reduced cytochrome b_5, since it undergoes rapid autoxidation forming superoxide radicals [37]. Cytochrome b_5 as the source of the inhibiting hydrogen peroxide, however, would implicate the existence of different rates of cytochrome b_5 reduction in the soluble and the amphiphilic systems, even though the cytochrome b_5 concentrations as well as the reductase activities were apparently the same. Since cytochrome b_5 reductase activities were determined in isolation using the artificial substrate ferricyanide, an alternative assay using cytochrome c as an electron acceptor was performed. In this assay, cytochrome c oxidizes cytochrome b_5 reduced by the action of cytochrome b_5 reductase. This test system thus gives a more useful estimate of the electron delivery rate from NADH to cytochrome b_5 catalysed by cytochrome b_5 reductase.

The use of this assay for determination of reductase activity revealed a significant difference between the amphiphilic and the soluble systems. Employing the same amounts of reductase as in the hydroxylase assays (3.6 μmol ferricyanide reduced min^{-1} per assay), the soluble reductase and soluble cytochrome b_5 exhibited a cytochrome c reduction rate of 32 nmol min^{-1} per assay, while the rate of cytochrome c reduction by the amphiphilic reductase with amphiphilic cytochrome b_5 was 142 nmol min^{-1} per assay. This suggests that the rate of electron transfer between the amphiphilic forms of cytochrome b_5 reductase and cytochrome b_5 is significantly greater than between the hydrophilic species. Since the consumption of reducing equivalents by the hydroxylase is very low (in the range of 0.1 $nmole^{-1}$ min^{-1} per assay), there is an excess of reduced cytochrome b_5 in both systems, which may undergo autoxidation and thus lead to production of superoxide radicals and subsequently hydrogen peroxide. In the amphiphilic system, due to a greater rate of cytochrome b_5 reduction, this effect is probably enhanced to a critical level, possibly leading to excess hydrogen peroxide formation and destruction of the CMP-Neu5Ac hydroxylase system. To support this, the amphiphilic system was assayed with varying amounts of cytochrome b_5 reductase. As shown in Fig. 5, linear hydroxylation rates were obtained at low reductase activities, whereas higher reductase activities led to the previously described inhibition of the system.

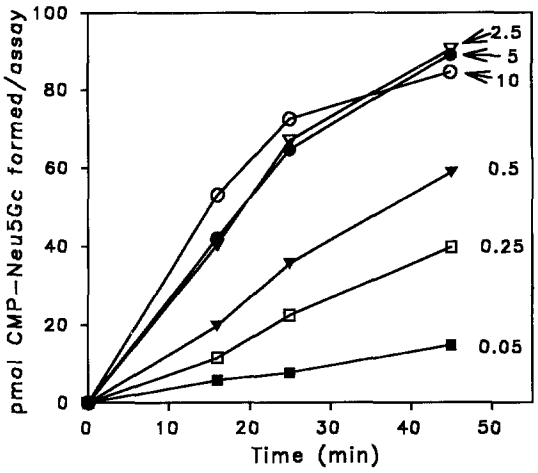

Figure 5. Dependence of hydroxylase turnover on cytochrome b_5 reductase activity. The time course of CMP-Neu5Ac hydroxylation was determined in a reconstituted system using 5 μM amphiphilic cytochrome b_5 and various amounts of amphiphilic cytochrome b_5 reductase. Reductase activities indicated (nmol min^{-1} per assay) were determined using the cytochrome c assay described in Materials and methods.

Although in the system reconstituted with mouse liver microsomes, and thus also with amphiphilic cytochrome b_5 and reductase, a cytochrome c reduction activity of 14 nmol min^{-1} per assay was used, the expected inhibition of the hydroxylase was not seen (Fig. 4a). This may be due to the presence of catalase, derived from contaminating peroxisomes, thus providing sufficient protection from hydrogen peroxide. Due to this protection mechanism, the use of solubilized microsomes in the assays performed during hydroxylase purification guaranteed a quantitative determination of the hydroxylase activities.

These results show, that the conditions for reconstitution of the hydroxylase system must be chosen carefully. An excess of reduced cytochrome b_5 should be avoided, since autoxidation results in accumulation of hydrogen peroxide and therefore inhibition of the system. This effect probably does not occur *in vivo*, since reduced cytochrome b_5 is oxidized by various cellular enzymes and many mechanisms for the removal of active oxygen exist within the cell.

Characterization of CMP-Neu5Ac hydroxylase in reconstituted systems

For a detailed characterization of CMP-Neu5Ac hydroxylase in reconstituted systems it is necessary to avoid the inactivation process described above. Therefore, cytochrome b_5 reductase activities were chosen, where systems reconstituted with 5 μM cytochrome b_5 were just sufficiently saturated. This occurred at total cytochrome c reductase activities of 3 nmol min^{-1} for the soluble reductase and

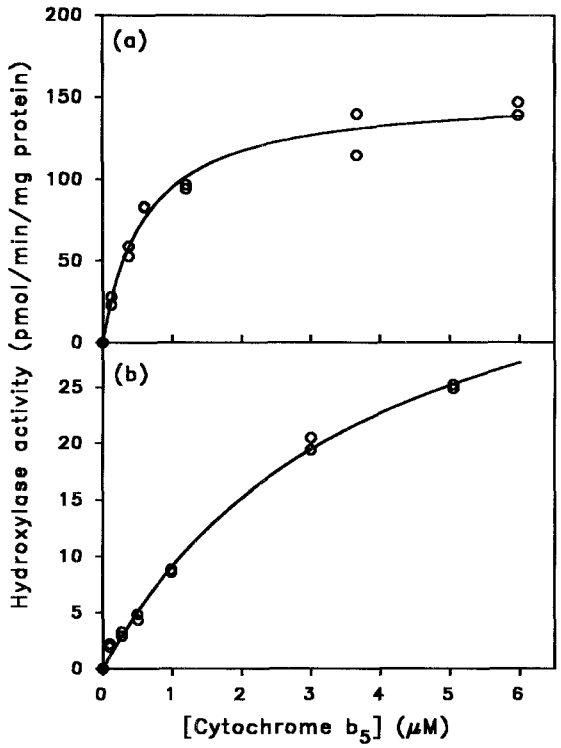

Figure 6. Dependence of the specific hydroxylase activity on cytochrome b_5 concentration. Hydroxylase systems were reconstituted using (a) soluble and (b) amphiphilic cytochrome b_5 reductase and various concentrations of the respective forms of cytochrome b_5. Cytochrome c reducing activities determined using the assay described in Materials and methods were 3 and 2.5 nmol min^{-1} for the soluble and the amphiphilic system, respectively. Specific hydroxylase activities are given with respect to the amount of hydroxylase protein present in the assays.

2.5 nmol min^{-1} for the amphiphilic reductase in the respective hydroxylase assays.

Hydroxylase activity was determined in soluble and amphiphilic systems with varying cytochrome b_5 concentrations, as shown in Fig. 6. K_M and V_{max} values of both systems were determined by non-linear regression using the program Enzfitter (Elsevier Science Publisher BV, Cambridge, UK), and the curves shown were calculated using these deduced parameters. The K_M values (±SD) were 0.6 (±0.08) μM for soluble cytochrome b_5 and 3.9 (±0.43) μM for the amphiphilic form. The V_{max} values of the soluble and amphiphilic systems were 153 (±6.0) and 45 (±2.6) nmol min^{-1} mg protein, respectively.

Since the interactions of both cytochrome b_5 forms with their reductases may also be influenced by the cytochrome b_5 concentration, the values determined can only be regarded as apparent K_Ms. Nevertheless, the low K_M values obtained for both cytochrome b_5 forms suggest that they have a high affinity for the hydroxylase. This may be an adaptation to the cytochrome b_5 concentration in the cell, thus ensuring efficient electron delivery to the hydroxylase and minimizing the formation of active oxygen species. A high affinity for cytochrome b_5 may also be necessary, since *in vivo* CMP-Neu5Ac hydroxylase is in competition with other cytochrome b_5-dependent enzymes.

The apparent higher affinity for the soluble cytochrome b_5 form in comparison with the amphiphilic form confirms the preference of the hydroxylase for soluble cytochrome b_5, as has been reported in studies on crude hydroxylase fractions [19]. These differing K_M values may reflect the less hindered access of soluble cytochrome b_5 to the hydroxylase, since the amphiphilic form is presumably associated with detergent aggregates in these experiments. Nevertheless, in mouse liver high-speed supernatant the amphiphilic form was found to predominate [19], suggesting that the preferential interaction with soluble cytochrome b_5 is not of physiological significance in this tissue.

To examine the kinetics with the CMP-Neu5Ac substrate, the hydroxylase was reconstituted either with Triton X-100-solubilized mouse liver microsomes or with both purified forms of cytochrome b_5 reductase and cytochrome b_5. The specific hydroxylase activities determined with various substrate concentrations are shown in Fig. 7. The respective apparent K_M and V_{max} values were determined as described above.

Apparent K_M values for CMP-Neu5Ac were 5.5 (±0.48) μM in the system reconstituted with microsomes, 8.8 (±0.83) μM with the amphiphilic system and 13 (±0.87) μM with the soluble system. The respective V_{max} values were 96 (±4.4), 84 (±4.7) and 172 (±7.6) nmol min^{-1} per mg protein.

The low apparent K_M values determined in all three systems indicate a high affinity of the hydroxylase for CMP-Neu5Ac and probably reflect a low steady-state concentration of this sugar nucleotide in the cell. Interestingly, the values obtained in this study were all significantly higher than the value of 1.4 μM reported previously for the hydroxylase in mouse liver high-speed supernatant [8]. However, in these latter experiments, the concentration of endogenous cytochrome b_5 severely limited the activity of the hydroxylase [19], possibly leading to an altered apparent K_M. In addition, the hydroxylase preparation used in the experiments described in this study resulted from a long purification procedure, which may have led to a reduced affinity for CMP-Neu5Ac. Nevertheless, the apparent K_M values determined here are comparable to those determined for the hydroxylase from starfish (18 μM), [17], pig submandibular glands (2.5 μM) [38] and rat small intestine (0.6 μM) [11].

Since in all three reconstituted systems the hydroxylation rates were limited by the substrate concentrations rather than by the electron delivery rates, similar apparent K_M and V_{max} values might have been expected. However, system-dependent differences in the kinetic parameters for CMP-Neu5Ac were observed. Recently, the hydroxylation of CMP-Neu5Ac was proposed to occur in a ternary complex consisting of cytochrome b_5, the hydroxylase and CMP-

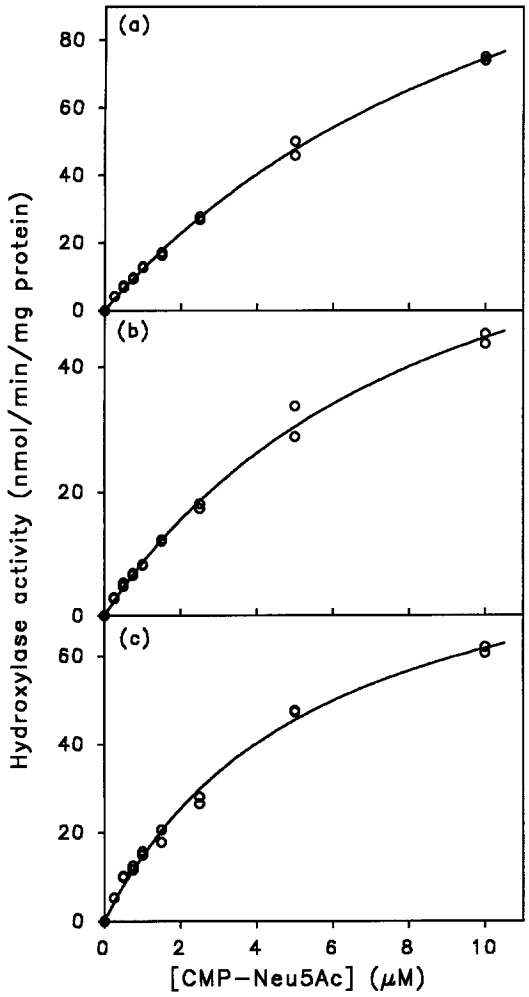

Figure 7. Dependence of the specific hydroxylase activity on CMP-Neu5Ac concentration. Hydroxylase systems were reconstituted using (a) soluble or (b) amphiphilic forms of cytochrome b_5 reductase and cytochrome b_5 or (c) Triton-X-100 solubilized mouse liver microsomes under the following conditions. Soluble system: cytochrome c reductase activity 3 nmol min^{-1}, 3 μM cytochrome b_5; amphiphilic system: cytochrome c reductase activity 2.5 nmol min^{-1}, 10 μM cytochrome b_5; microsomes: cytochrome c reductase activity 6 nmol min^{-1}. Specific hydroxylase activities are given with respect to the amount of hydroxylase protein present in the assays.

Neu5Ac [39]. It is thus conceivable, that the type of cytochrome b_5 participating in this complex may influence the kinetics of CMP-Neu5Ac hydroxylation, as may be reflected by the different apparent K_M values obtained for CMP-Neu5Ac with soluble and amphiphilic forms of cytochrome b_5.

The results obtained in this study clearly demonstrate that the turnover by the hydroxylase is the rate limiting step in the whole catalytic process. For example, in experiments with the soluble system, the potential rate of electron delivery from cytochrome b_5, as measured by the cytochrome c reductase assay, was 3 nmol min^{-1} per assay, while the maximal rate of CMP-Neu5Ac hydroxylation was 8.6 pmol min^{-1} per assay. The utilization of the cytochrome b_5 reductase/cytochrome b_5 system by the hydroxylase thus does not seem to be very effective in such isolated systems.

Finally, the results in this report may point to mechanisms involved in regulating the incorporation of Neu5Gc into glycoconjugates. The characteristic ratios of Neu5Gc and Neu5Ac in the glycoconjugates of developing and adult tissues suggest that particular sialic acid patterns may be of biological importance and thus their generation must be under strict control. Since cytochrome b_5 is involved in a number of metabolic processes, it is unlikely, that the control of sialic acid hydroxylation would depend on this electron donor. The low apparent K_M values of the hydroxylase for CMP-Neu5Ac and cytochrome b_5 may ensure that the turnover of the hydroxylase *in vivo* is kept at an optimal level. A regulation of CMP-Neu5Ac hydroxylation and thus of the ratio of glycoconjugate-bound Neu5Ac and Neu5Gc might occur by varying the amount of hydroxylase protein within the cell, possibly by controlling the expression of the hydroxylase gene.

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References

1. Reuter G, Schauer R (1994) *Methods Enzymol* **230**:168–99.
2. Schauer R (1985) *Trends Biochem Sci* **10**:357–60.
3. Varki A (1992) *Glycobiology* **2**:25–40.
4. Corfield AP, Schauer R (1982) *Cell Biol Monogr* **10**:5–55.
5. Higashi H, Ikuta K, Ueda S, Kato S, Hirabayashi Y, Matsumoto M, Naiki M (1984) *J Biochem* **59**:785–94.
6. Higashi H, Hirabayashi Y, Fukui Y, Naiki M, Matsumoto M, Ueda S, Kato S (1985) *Cancer Res* **45**:3796–802.
7. Shaw L, Schauer R (1988) *Biol Chem Hoppe-Seyler* **369**:477–86.
8. Shaw L, Schauer R (1989) *Biochem J* **263**:355–63.
9. Lepers A, Shaw L, Schneckenburger P, Cacan R, Verbert A, Schauer R (1990) *Eur J Biochem* **193**:715–23.
10. Shaw L, Yousefi S, Dennis JW, Schauer R (1991) *Glycoconjugate J* **8**:434–41.
11. Bouhours J-F, Bouhours D (1989) *J Biol Chem* **264**:16992–4.
12. Muchmore EA (1992) *Glycobiology* **2**:337–43.
13. Kawano T, Kozutsumi Y, Takematsu H, Kawasaki T, Suzuki A (1993) *Glycoconjugate J* **10**:109–15.
14. Kozutsumi Y, Kawano T, Yamakawa T, Suzuki A (1990) *J Biochem* **108**:704–6.

15. Shaw L, Schneckenburger P, Carlsen J, Christiansen K, Schauer R (1992) *Eur J Biochem* **206**:269–77.
16. Kozutsumi Y, Kawano T, Kawasaki H, Suzuki K, Yamakawa T, Suzuki A (1991) *J Biochem* **110**:429–35.
17. Schlenzka W, Shaw L, Schauer R (1993) *Biochim Biophys Acta* **1161**:131–8.
18. Schneckenburger P, Shaw L, Carlsen J, Christiansen K, Schauer R (1993) *Glycoconjugate J* **10**:329.
19. Shaw L, Schneckenburger P, Schlenzka W, Carlsen J, Christiansen K, Jürgensen D, Schauer R (1994) *Eur J Biochem* **219**:1001–11.
20. Schlenzka W, Shaw L, Schauer R (1993) *Glycoconjugate J* **10**:329.
21. Arinç E (1991) In *Molecular Aspects of Monooxygenases and Bioactivation of Toxic Compounds*, NATO ASI Series A: Life Sciences vol. 202 (Arinç E, Schenkman JB, Hodgson E, eds) pp. 149–70. New York and London: Plenum Press.
22. Spatz L, Strittmatter P (1973) *J Biol Chem* **248**:793–9.
23. Tajima S, Enomoto K, Sato R (1987) *J Biochem* **84**:1573–86.
24. Mitoma J, Ito A (1992) *EMBO J* **11**:4197–203.
25. Passon PB, Reed DW, Hultquist DE (1972) *Biochim Biophys Acta* **275**:62–73.
26. Kawano T, Kozutsumi Y, Kawasaki T, Suzuki A (1993) *Glycoconjugate J* **10**:331.
27. Schneckenburger P, Shaw L, Schauer R (1993) *Biol Chem Hoppe-Seyler* **374**:956.
28. Williams CH (1976) In *The Enzymes* 3rd edition vol XIII, part C (Boyer PD, ed.) pp. 89–173. New York: Academic Press.
29. Masters BSS, Williams CH, Kamin H (1967) *Methods Enzymol* **10**:565–73.
30. Margoliash E, Frohwirt N (1959) *Biochem J* **71**:570–2.
31. Hames BD, Rickwood D (eds) (1981) *Gel Electrophoresis of Proteins: A Practical Approach*. London and Washington DC: IRL Press Ltd.
32. Heukeshoven J, Dernik R (1985) *Electrophoresis* **6**:103–12.
33. Schlenzka W, Shaw L, Schauer R (1993) *Biol Chem Hoppe-Seyler* **374**:955.
34. Lever M (1977) *Anal Biochem* **83**:274–84.
35. Halliwell B, Gutteridge JMC (1984) *Biochem J* **219**:1–14.
36. Archakov AI, Bachmanova GI (eds) (1990) In *Cytochrome P-450 and Active Oxygen* pp. 4–14. Taylor & Francis, London.
37. Berman MC, Adnamis CM, Ivanetich KM, Kench JE (1976) *Biochem J* **157**:237–46.
38. Muchmore EA, Milewski M, Varki A, Diaz S (1989) *J Biol Chem* **264**:20216–23.
39. Takematsu H, Kawano T, Koyama S, Kozutsumi Y, Suzuki A, Kawasaki T (1994) *J Biochem* **115**:381–6.