Structural requirements for the utilization of ascorbate analogues in the prolyl 4-hydroxylase reaction

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The ability of structural analogues of ascorbate to serve as substitutes for this reducing agent in the prolyl 4-hydroxylase reaction was studied. In experiments using the purified enzyme, variations of the compounds' side chain were compatible with co-substrate activity. The presence of very large hydrophobic substituents or a positively charged group caused an increase in the observed K_m values. A negative charge and smaller modifications did not change the affinity to the enzyme when compared with L-ascorbate. 6-Bromo-6-deoxy-L-ascorbate had a lower K_m than the physiological reductant. Substitution at the -OH group in ring position 3 prevented binding to the enzyme. The same pattern of activity was observed when the full and uncoupled prolyl 4-hydroxylase reactions were studied. The $V_{max.}$ values with all compounds were similar. The reaction of microsomal prolyl 4-hydroxylase was supported by D-isoascorbate, O^6-tosyl-L-ascorbate and 5-deoxy-L-ascorbate, giving the same dose–response behaviour as L-ascorbate itself. Again, 6-bromo-6-deoxy-L-ascorbate gave a lower K_m and a similar $V_{max.}$ value. L-Ascorbic acid 6-carboxylate produced substrate inhibition at concentrations above 0.3 mM. The K_m and $V_{max.}$ values calculated from concentrations up to 0.2 mM were similar to those of L-ascorbate. The enzyme activity observed with 6-amino-6-deoxy-L-ascorbate was very low in the microsomal hydroxylation system. The calculated $V_{max.}$ value was lower than that of L-ascorbate, suggesting a restriction of the access of this compound to the enzyme.

INTRODUCTION

Prolyl 4-hydroxylase [procollagen-L-proline,2-oxoglutarate: oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2] catalyses the hydroxylation of peptide-bound proline residues in peptides of the sequence -Xaa-Pro-Gly- [1–3]. The active enzyme is an $\alpha_2\beta_2$ tetramer. Its β-subunit was identified as being identical with the enzyme protein disulphide-isomerase (EC 5.3.4.1) [4]. The thermal stability of the triple helix of collagens and related proteins, and thus their structure and function, is crucially dependent upon the intramolecular hydrogen bonds involving the 4-hydroxyproline residues synthesized by prolyl 4-hydroxylase.

The first step of the enzymic reaction consists of the oxidative decarboxylation of 2-oxoglutarate by one atom of molecular oxygen. This process has been suggested to proceed as a ligand reaction in the co-ordination sphere of enzyme-bound Fe^{2+} [5], generating succinate, CO_2 and a highly reactive iron–oxygen-atom complex, a ferryl ion, which subsequently hydroxylates an appropriate proline residue, probably by an abstraction–recombination mechanism [5]. The generation of the ferryl ion can proceed without subsequent hydroxylation in so-called uncoupled reaction cycles [6–8]. Ascorbate is utilized as a specific alternative acceptor of the ferryl oxygen in these reaction cycles [8,9]. In the absence of this reducing agent, prolyl 4-hydroxylase is rapidly inactivated by self-oxidation [10,11].

Because the function of ascorbate in the prolyl 4-hydroxylase reaction had remained obscure for a long time, little is known about the structure–activity relationship of this co-substrate. The side chain, consisting of C-5 and C-6 of the molecule, apparently contributes very little to the binding [12]. Systematic studies do not exist.

In the present study we investigated the requirements for binding of this co-substrate to prolyl 4-hydroxylase *in vitro*, as well as the requirements for access to the microsomal enzyme, by using structurally modified ascorbate derivatives (Figure 1).

MATERIALS AND METHODS

Compounds and reagents

The ascorbate analogues used in the present study (Figure 1) were synthesized as described previously [13–18]. The synthetic substrate for prolyl 4-hydroxylase, $(Pro\text{-}Pro\text{-}Gly)_{10},9H_2O$, was obtained from the Protein Research Foundation (Minoh, Osaka, Japan). Catalase and poly-(L-proline) of M_r approx. 5500 were purchased from Sigma (St. Louis, MO, U.S.A.). 2-Oxo[1-^{14}C]glutarate was obtained from Amersham International (Amersham, Bucks., U.K.). It was diluted to 100000 d.p.m./0.1 μmol by mixing with unlabelled compound purchased from Fluka (Buchs, Switzerland). D-Isoascorbate was obtained from Sigma (Munich, Germany).

Prolyl 4-hydroxylase was purified from homogenized 14-day-old chick embryos by $(NH_4)_2SO_4$ fractionation (0–70% satn.), affinity chromatography on poly-(L-proline)–Sepharose 4B, DEAE-cellulose chromatography and gel filtration as previously reported [19,20]. The enzyme was pure as judged by SDS/PAGE. Microsomes containing prolyl 4-hydroxylase and [^{3}H]proline-labelled underhydroxylated procollagen were purified from chicken-embryo bones in the presence of 0.5 mM 2,2′-dipyridyl [21]. The intactness of the microsomes was controlled as described previously [21]. After their preparation, the microsomes were stored frozen in liquid N_2.

Abbreviation used: DMSO, dimethyl sulphoxide.

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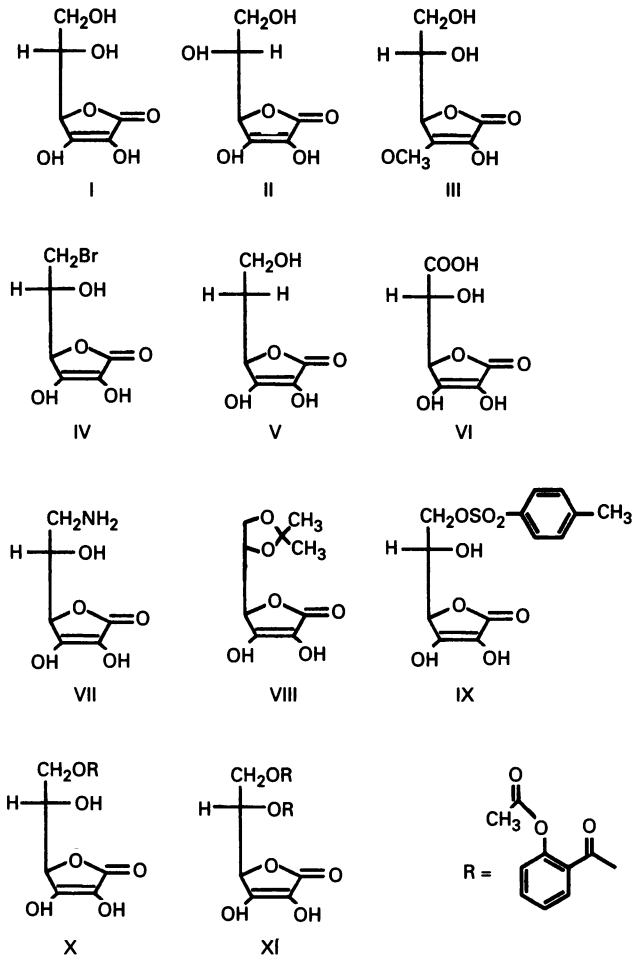

Figure 1 Structures of ascorbate analogues used in the present study

The compounds are: (I) L-ascorbate, (II) D-isoascorbate, (III) 3-methoxy-L-ascorbate, (IV) 6-bromo-6-deoxy-L-ascorbate (synthesized as described in [16]), (V) 5-deoxy-L-ascorbate [14], (VI) L-ascorbic acid 6-carboxylate [17,18], (VII) 6-amino-6-deoxy-L-ascorbate [15], (VIII) O^5O^6-isopropylidene-L-ascorbate [12], (IX) O^6-tosyl-L-ascorbate [17], (X) O^6-(2-acetoxybenzoyl)-L-ascorbate [17] and (XI) O^5O^6-bis-(2-acetoxybenzoyl)-L-ascorbate [17].

Assays of enzyme activity

The ability of the ascorbate analogues to support the activity of purified prolyl 4-hydroxylase was studied by incubation of 0.05–0.2 μg of enzyme in 1 ml of 0.05 M Tris/HCl, pH 7.5, containing 0.05 mM $FeSO_4$, 0.1 mg of $(Pro\text{-}Pro\text{-}Gly)_{10}$ and 0.1 mM 2-oxo[1-^{14}C]glutarate (60000 d.p.m.), 0.1 mg of catalase, 0.1 mM dithiothreitol and 2 mg of BSA, as well as ascorbate or its analogues at various concentrations. The tubes were then sealed, kept at 37 °C for 30 min, and the amount of $^{14}CO_2$ produced was determined as described previously [19]. In the experiments studying the uncoupled prolyl 4-hydroxylase reaction, the concentration of enzyme was raised to 5 μg/ml, because the uncoupled decarboxylation of 2-oxoglutarate is known to proceed at only about 1–2% of the $V_{max.}$ of the full reaction. $(Pro\text{-}Pro\text{-}Gly)_{10}$ was replaced by poly-(L-proline), a peptide that is not itself hydroxylated, but is known to enhance the uncoupled reaction [6–8]. All other conditions were identical with those used in the complete assay. In all experiments, parallel determinations of enzyme activity of L-ascorbate analogues and the parent compound were carried out in order to allow the calculation of relative $V_{max.}$ values.

Owing to the low solubility of O^5O^6-bis-(2-acetoxybenzoyl)-L-ascorbate in water, stock solutions of this compound were prepared in dimethyl sulphoxide (DMSO). In the test, DMSO concentrations did not exceed 10% of the total volume. Identical amounts of DMSO were added to the control and L-ascorbate containing tubes to compensate any effect of this substance upon enzyme activity.

Microsomal prolyl 4-hydroxylase was assayed by suspending aliquots of thawed microsomes in 0.25 M sucrose/1 mM $MgCl_2$/5 mM KCl/0.05 M Tris/HCl, pH 7.6. They were incubated for 20 min at 37 °C in a reaction mixture containing 0.1 mM 2-oxoglutarate, 0.2-mM $FeSO_4$, 0.1 mM-thymol, 0.1 mM-dithiothreitol and various concentrations of L-ascorbate or ascorbate analogues in a total volume of 0.1 ml. The amount of released ^3H_2O was measured as described previously [22]. Controls incubated with various concentrations of L-ascorbate were used in every experiment in order to allow the direct comparison of $V_{max.}$ values. All experiments were conducted three times.

Calculation of results

K_m and $V_{max.}$ values were calculated from Lineweaver–Burk plots of the original data. L-Ascorbate concentrations of 0.05–0.5 mM in the experiments using purified prolyl 4-hydroxylase and 0.04–0.8 mM in the microsomal hydroxylation system were used for calculation. Concentrations outside these ranges produced a non-linear behaviour of the Lineweaver–Burk transformations. The concentrations of the ascorbate analogues were adjusted to their respective active concentrations.

RESULTS

Purified-enzyme studies

In the experiments using purified prolyl 4-hydroxylase, a clear structure–activity relationship could be deduced from the systematic variations imposed on the ascorbate molecule. All the changes introduced into the side chain of L-ascorbate produced compounds that supported enzymic activity, although some differences regarding the K_m values were observed. These data are summarized in Table 1. The calculated relative $V_{max.}$ values showed some variation, but their means were close to those of L-ascorbate determined in parallel experiments (results not shown).

The introduction of one large substituent was well tolerated. O^6-Tosyl- and O^6-acetoxybenzoyl-L-ascorbate, as well as 5-deoxy-L-ascorbate all had K_m values which were not significantly different from that of ascorbate itself in the full reaction. The introduction of two large substituents, as in O^5O^6-bis-(2-acetoxybenzoyl)-L-ascorbate, resulted in a reduction of the affinity for the enzyme.

The affinity of 6-amino-6-deoxy-L-ascorbate to the enzyme was decreased 2–4-fold when compared with that of L-ascorbate, as judged from the compound's K_m value. The K_m value of L-ascorbic acid 6-carboxylate differed from that of L-ascorbate only in the full reaction. The most active substance was 6-bromo-6-deoxy-L-ascorbate. Its K_m values in the full and uncoupled reactions were as low as 0.14 and 0.09 mM respectively and thus only about half of the value of the parent compound.

The K_m value obtained for ascorbate in the uncoupled reaction of prolyl 4-hydroxylase was significantly lower than that obtained in the full reaction. In general, the pattern of co-substrate activity of the ascorbate analogues was the same as in the full reaction, although all compounds gave lower K_m values. This was also true

Table 1 Structure–activity relationship of ascorbate analogues in the full and uncoupled reactions catalysed by prolyl 4-hydroxylase

The experiments were carried out as described in the Materials and methods section. Values in parentheses refer to the number of experiments performed. In those cases where duplicate determinations were carried out, the values obtained in the individual experiments are given. Statistical significance: $^{*}P$ and $^{**}P$, < 0.05 and < 0.01 respectively versus L-ascorbate in the full reaction of purified prolyl 4-hydroxylase; $^{\$}P$, $<$ and $^{\$\$}P$, < 0.05 and 0.01 respectively versus L-ascorbate in the uncoupled reaction of prolyl 4-hydroxylase.

	K_m (mM)	
Compound	Full reaction	Uncoupled reaction
L-Ascorbate	0.37 ± 0.15 ($n = 19$)	0.25 ± 0.12 ($n = 18$)*
D-Isoascorbate	0.3 ([12])	0.16 ± 0.04 ($n = 3$)
5-Deoxy-L-ascorbate	0.23 ± 0.1 ($n = 3$)	0.14, 0.21
6-Bromo-6-deoxy-L-ascorbate	0.14 ± 0.04 ($n = 5$)**	0.09 ± 0.015 ($n = 4$)$^{\$}$
6-Amino-6-deoxy-L-ascorbate	1.6 ± 0.67 ($n = 9$)**	0.54, 0.61$^{\$\$}$
L-Ascorbic acid 6-carboxylate	0.65 ± 0.17 ($n = 6$)**	0.26 ± 0.14 ($n = 4$)
O^6-Tosyl-L-ascorbate	0.26 ± 0.11 ($n = 3$)	0.15, 0.17
O^6-Acetoxybenzoyl-L-ascorbate	0.41 ± 0.07 ($n = 3$)	0.39 ± 0.1 ($n = 4$)$^{\$}$
O^5O^6-Bis-(acetoxybenzoyl)-L-ascorbate	1.9 ± 0.28 ($n = 3$)**	1.73 ± 0.48 ($n = 6$)$^{\$\$}$
O^5O^6-Isopropylidene-L-ascorbate	0.36 ([12])	0.3, 0.38
3-Methoxy-L-ascorbate	Not active	Not active
3-Benzoxy-L-ascorbate	Not active	Not active

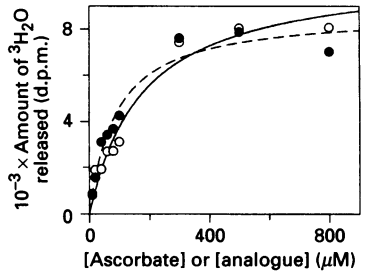

Figure 2 Microsomal prolyl 4-hydroxylase activity supported by 5-deoxy-L-ascorbate

The enzyme activity supported by 5-deoxy-L-ascorbate (●) and L-ascorbate (○) were determined in a parallel experiment. The $V_{max.}$ values, calculated from Lineweaver–Burk plots, were 7930 and 9525 d.p.m. respectively. This Figure shows the result of a typical experiment.

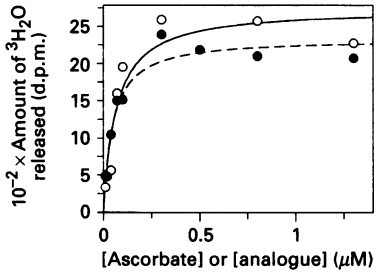

Figure 3 Microsomal prolyl 4-hydroxylase activity supported by D-isoascorbate

The enzyme activity supported by D-isoascorbate (●) and L-ascorbate (○) were determined in a parallel experiment. The $V_{max.}$ values, calculated from Lineweaver–Burk plots, were 4370 and 2160 d.p.m. respectively. This Figure shows the result of a typical experiment.

for D-isoascorbate and O^5O^6-isopropylidene-L-ascorbate, two compounds which have been previously studied in the full reaction [12,23,24].

By contrast, substituents in ring position 3, such as benzyloxy and methoxy groups, completely abolished co-substrate activity.

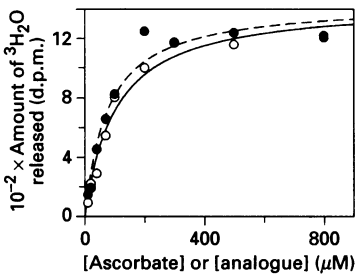

Figure 4 Microsomal prolyl 4-hydroxylase activity supported by O^6-tosyl-L-ascorbate

The enzyme activity supported by O^6-tosyl-L-ascorbate (●) and L-ascorbate (○) were determined in a parallel experiment. The $V_{max.}$ values, calculated from Lineweaver–Burk plots, were 20290 and 20370 d.p.m. respectively. This Figure shows the result of a typical experiment.

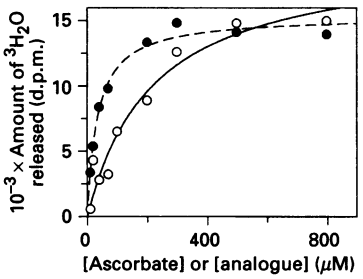

Figure 5 Microsomal prolyl 4-hydroxylase activity supported by 6-bromo-6-deoxy-L-ascorbate

The enzyme activity supported by 6-bromo-6-deoxy-L-ascorbate (●) and L-ascorbate (○) were determined in a parallel experiment. The $V_{max.}$ values, calculated from Lineweaver–Burk plots, were 17900 and 21600 d.p.m. respectively. This Figure shows the result of a typical experiment.

These substances did not act as inhibitors of the enzymic reaction in the presence of the physiological substrate L-ascorbate either, demonstrating that they are not bound to the enzyme's active

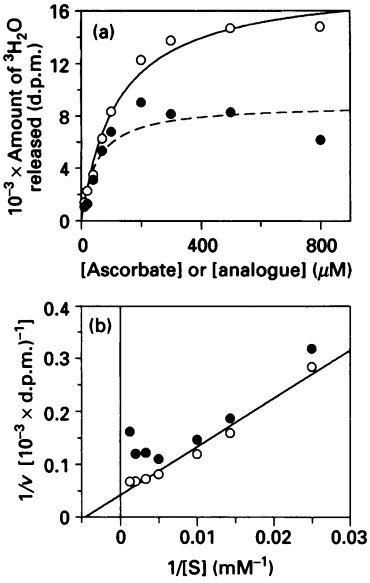

Figure 6 Microsomal prolyl 4-hydroxylase activity supported by L-ascorbic acid 6-carboxylate

(**a**) Plot showing the original data; (**b**) Lineweaver–Burk plot. The enzyme activity supported by L-ascorbic acid 6-carboxylate (●) and L-ascorbate (○) were determined in a parallel experiment. In spite of the substrate inhibition observed at concentrations exceeding 0.2 mM of L-ascorbic acid 6-carboxylate, the K_m values of ascorbate and the co-substrate analogue were almost identical (0.227 mM for L-ascorbic acid 6-carboxylate and 0.216 mM for L-ascorbate) in this individual experiment if only the concentrations below 0.2 mM were used for the calculation of the L-ascorbic acid 6-carboxylate (**b**). The obtained $V_{max.}$ values were 21 300 and 23 600 d.p.m. respectively. This Figure shows the result of a typical experiment.

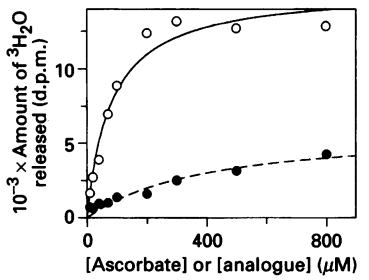

Figure 7 Microsomal prolyl 4-hydroxylase activity supported by 6-amino-6-deoxy-L-ascorbate

The enzyme activity supported by 6-amino-6-deoxy-L-ascorbate (●) and L-ascorbate (○) were determined in a parallel experiment. The $V_{max.}$ values obtained were 4900 and 21 200 d.p.m. respectively. This Figure shows the result of a typical experiment.

site. The compound 2-methoxy-L-ascorbate showed some co-substrate activity, but this was due to instability with respect to hydrolytic cleavage in aqueous solutions at pH 7.6, generating free L-ascorbate (results not shown).

Studies involving microsomal prolyl 4-hydroxylase

The ability of selected compounds to support the activity of microsomal prolyl 4-hydroxylase was studied. The K_m of L-ascorbate in this system was found to be 0.21 ± 0.07 mM ($n = 14$), and thus significantly lower than the K_m of ascorbate in the full reaction of the purified enzyme. All ascorbate analogues

Table 2 Activity of ascorbate and its analogues in the microsomal hydroxylation system

The experiments were carried out as described in Materials and methods section. All experiments using ascorbate analogues were carried out in triplicate. Statistical significance: $^{**}P$, < 0.01 versus L-ascorbate in the full reaction of purified prolyl 4-hydroxylase; $^{\$\$}P$, < 0.01 versus L-ascorbate in the microsomal hydroxylation system. All compounds except 6-amino-6-deoxy-L-ascorbate gave similar $V_{max.}$ values compared with that of L-ascorbate.

Compound	K_m (mM)
L-Ascorbate	0.21 ± 0.07 ($n = 14$)**
D-Isoascorbate	0.19 ± 0.1
5-Deoxy-L-ascorbate	0.21 ± 0.12
6-Bromo-6-deoxy-L-ascorbate	$0.04 \pm 0.0065$$^{\$\$}$
6-Amino-6-deoxy-L-ascorbate	0.24 ± 0.095
L-Ascorbic acid 6-carboxylate	0.19 ± 0.03
O^6-Tosyl-L-ascorbate	0.25 ± 0.095

supported the activity of microsomal prolyl 4-hydroxylase, and three of them, i.e., 5-deoxy-L-ascorbate, D-isoascorbate, and O^6-tosyl-L-ascorbate gave dose–response curves indistinguishable from those of L-ascorbate itself (Figures 2–4; Table 2). As with purified prolyl 4-hydroxylase, 6-bromo-6-deoxy-L-ascorbate had a lower K_m than L-ascorbate itself; the observed $V_{max.}$ was similar to that of ascorbate (Figure 5). L-Ascorbic acid 6-carboxylate supported enzyme activity, but substrate inhibition was observed at concentrations exceeding 0.2 mM. The K_m and $V_{max.}$ values calculated from the concentrations between 0 and 0.2 mM were comparable with those of L-ascorbate, determined in parallel experiments (Figures 6a and 6b). The enzyme activity obtained with 6-amino-6-deoxy-L-ascorbate was very low (Figure 7). The calculated K_m value was similar to that of L-ascorbate (Table 2), but $V_{max.}$ amounted to only 23 % of the reaction supported by L-ascorbate.

DISCUSSION

The results presented here demonstrate that purified prolyl 4-hydroxylase is able to accept a number of ascorbate analogues instead of the physiological substrate, if the two hydroxy groups participating in the redox reaction remain unchanged. Compounds with substitutions at the *ring*-hydroxy groups neither support the enzymic reaction nor do they act as inhibitors. This finding suggests a direct participation of the ring hydroxy groups in the binding of this co-substrate, in agreement with a previous study [12]. By contrast, hydroxy groups located in the side chain of ascorbate are neither necessary for binding of this co-substrate to the enzyme's active site nor for its transport through the microsomal membrane. The presence of a positive charge in the side chain in the 6-amino-6-deoxy-L-ascorbate molecule prevents its effective utilization by the enzyme. It apparently also interferes with the compound's permeation through the endoplasmic membrane, resulting in a decreased $V_{max.}$ in the microsomal hydroxylation assay. Steric restrictions in the side-chain binding domain of prolyl 4-hydroxylase are little. The side-chain binding domain appears to be of hydrophobic nature, because the binding of analogues containing both negatively and positively charged groups is impaired.

Comparison of the K_m values obtained in the full and uncoupled reactions shows consistently lower values in the uncoupled reaction. At present it cannot be ruled out that this effect is due to the increase of the enzyme concentration in the reaction mixture, which is necessary because the uncoupled reaction proceeds with only 1–2 % of the velocity of the full reaction [6–8].

A lower K_m for L-ascorbate was also observed in the microsomal hydroxylation assay when compared with its K_m in the full reaction. This finding is in agreement with those of a previous study suggesting intramicrosomal accumulation of L-ascorbate [25].

This work was supported in part by grants from the Academy of Finland Medical Research Council. During the course of the study, V.G. was the recipient of a long-term fellowship from EMBO, Heidelberg, Germany. We gratefully acknowledge the expert technical assistance of M. Seppänen and M. Weier.

REFERENCES

1 Kivirikko, K. I., Helaakoski, T., Tasanen, K., Vuori, K., Myllylä, R., Parkkonen, T. and Pihlajaniemi, T. (1990) Ann. N.Y. Acad. Sci. **580**, 132–142
2 Pihlajaniemi, T., Myllylä, R. and Kivirikko, K. I. (1991) J. Hepatol. **13**, Suppl. 3, S2–S7
3 Kivirikko, K. I., Myllylä, R. and Pihlajaniemi, T. (1992) in Focus on Post-Translational Modifications of Proteins (Harding, J. J. and Crabbe, M. J. C., eds.), pp. 1–51, CRC Press, Boca Raton, FL
4 Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M. L., Koivu, J. and Kivirikko, K. I. (1987) EMBO J. **6**, 643–649
5 Hanauske-Abel, H. M. and Günzler, V. (1982) J. Theor. Biol. **94**, 421–455
6 Rao, N. V. and Adams, E. (1978) J. Biol. Chem. **253**, 6327–6330
7 Counts, D. F., Cardinale, G. J. and Udenfriend, S. (1980) Proc. Natl. Acad. Sci. U.S.A. **75**, 2145–2149
8 Myllylä, R., Majamaa, K., Günzler, V., Hanauske-Abel, H. M. and Kivriikko, K. I. (1984) J. Biol. Chem. **259**, 5403–5405
9 de Jong, L. and Kemp, A. (1984) Biochim. Biophys. Acta **787**, 105–111
10 Myllylä, R., Kuutti-Savolainen, E. R. and Kivirikko, K. I. (1978) Biochem. Biophys. Res. Commun. **83**, 441–448
11 de Jong, L., Albracht, S. P. J. and Kemp, A. (1982) Biochim. Biophys. Acta **704**, 326–332
12 Majamaa, K., Günzler, V., Hanauske-Abel, H. M., Myllylä, R. and Kivirikko, K. I. (1986) J. Biol. Chem. **261**, 7819–7823
13 Dallacker, F. and Sanders, J. (1985) Chem. Ztg. **109**, 197–202
14 Dallacker, F. and Sanders, J. (1985) Chem. Ztg. **109**, 277–280
15 Dallacker, F. and Sanders, J. (1985) Chem. Ztg. **109**, 341–343
16 Bock, K., Lundt, I. and Pedersen, C. (1979) Carbohydr. Res. **68**, 313–319
17 Eisbach, R. (1991) Ph.D. Thesis, Rheinisch-Westfälische Technische Hochschule, Aachen
18 Stuber, H. A. and Tolbert, B. M. (1978) Carbohydr. Res. **60**, 251–258
19 Tuderman, L., Kuutti-Savolainen, E. R. and Kivirikko, K. I. (1975) Eur. J. Biochem. **52**, 9–16
20 Kedersha, N. L. and Berg, R. A. (1981) Collagen Rel. Res. **1**, 345–353
21 Tschank, G., Hanauske-Abel, H. M. and Peterkofsky, B. (1988) Arch. Biochim. Biophys. **261**, 312–323
22 Mata, J. M., Assad, R. and Peterkofsky, B. (1981) Arch. Biochem. Biophys. **206**, 93–104
23 Kivirikko, K. I. and Prockop, D. J. (1967) J. Biol. Chem. **242**, 4007–4012
24 Hutton, J. J., Tappel, A. L. and Udenfriend, S. (1967) Arch. Biochem. Biophys. **118**, 231–240
25 Peterkofsky, B., Tschank, G. and Luedke, C. (1988) Arch. Biochem. Biophys. **254**, 282–289

Received 4 November 1993; accepted 14 December 1993