in this study. The assistance of M. Bannon and J. Mouning in preparing the manuscript is also appreciated.

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Processive Action of the Two Peptide Binding Sites of Prolyl 4-Hydroxylase in the Hydroxylation of Procollagen

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Received May 29, 1987; Revised Manuscript Received September 14, 1987

ABSTRACT: The number of peptide binding sites of prolyl 4-hydroxylase was manipulated with the peptide photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$, and the effect on hydroxylation of the relatively short peptide substrate (Pro-Pro-Gly)$_5$ and of the long natural substrate procollagen was studied. With (Pro-Pro-Gly)$_5$ as a substrate, a linear relation was found between enzyme activity and the amount of covalently bound photoaffinity label, approximately 50% inactivation being reached at 1 mol of label/mol of enzyme. No difference in K_m value for (Pro-Pro-Gly)$_5$ was detected between unlabeled and partially labeled enzyme preparations. These results indicate that enzyme molecules with only one free active site hydroxylated the synthetic substrate (Pro-Pro-Gly)$_5$ with the same K_m and at half the rate of native enzyme. In contrast, with procollagen as a substrate a 5–10-fold increase in K_m was found with the fraction of enzyme containing only one free active site, as compared to the K_m for procollagen with nonlabeled enzyme. This finding is explained by an enzyme–kinetic model based on a processive action of the two peptide substrate binding sites of prolyl 4-hydroxylase, preventing dissociation of the enzyme–substrate complex between successive hydroxylations of a long peptide with multiple substrate sites. Such a mechanism leads to a low K_m for a long peptide by overcoming the diffusional constraints on the rate of association between the enzyme and the individual substrate sites.

Prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate:oxygen oxidoreductase, 4-hydroxylating, EC 1.14.11.2) catalyzes the 4-hydroxylation of proline residues located N-terminally of glycine in nascent pro-α chains of procollagen. This posttranslational modification is pivotal for the formation of the triple helix structure in the various col-

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lagens [for a review see Kivirikko and Myllylä (1980)]. The isolated enzyme is also able to hydroxylate proline residues in synthetic peptides. It is a long known but poorly understood phenomenon that the K_m of a peptide substrate strongly depends on the chain length of the peptide (Kivirikko & Prockop, 1967; Hutton et al., 1968; Kivirikko et al., 1972a). This property is shared by prolyl 4-hydroxylase with prolyl 3-hydroxylase (Risteli et al., 1977) and lysyl hydroxylase (Kivirikko et al., 1972b), two other members of the family of 2-oxoglutarate-dependent dioxygenases (Udenfriend & Cardinale, 1982) involved in the posttranslational modification of procollagen. Despite the large difference in affinity for procollagen compared to the shorter synthetic substrates, the k_{cat} of prolyl 4-hydroxylase is essentially the same for both (Berg & Prockop, 1973). Also, the inhibition of prolyl 4-hydroxylase by poly(L-proline) is dependent on the chain length of the inhibitor (Prockop & Kivirikko, 1969).

The phenomenon that the K_m decreases much more than proportionally with the length of a peptide substrate can be partly explained by the presence of a large peptide binding site, in which binding subsites are located adjacent to the catalytic binding site (Berg et al., 1977; de Waal et al., 1985). A large peptide binding site cannot, however, explain the large difference in K_m for pro-α chains of procollagen as compared to, for instance, $(Pro\text{-}Pro\text{-}Gly)_{20}$, the largest synthetic peptide substrate studied. The K_m for procollagen is 3 orders of magnitude lower than the K_m for $(Pro\text{-}Pro\text{-}Gly)_{20}$ when expressed per peptide. This difference cannot be explained by the fact that the procollagen has more substrate sites (approximately 100 per pro-α chain) than $(Pro\text{-}Pro\text{-}Gly)_{20}$, for the difference remains 250-fold when expressed per hydroxylatable peptidyl proline (Kivirikko & Myllylä, 1980). The length of $(Pro\text{-}Pro\text{-}Gly)_{20}$ (18 nm in its extended form) exceeds the dimensions of the enzyme, which is at most 10 nm in diameter (Olsen et al., 1973). All the available binding subsites are therefore expected to be occupied by $(Pro\text{-}Pro\text{-}Gly)_{20}$, implying that a collision of the enzyme with a still larger peptide substrate will not have a higher chance of resulting in an enzyme–substrate complex. As a consequence, the association rate constant k_a for $(Pro\text{-}Pro\text{-}Gly)_{20}$ is the highest possible, as far as the geometry of the binding site and the substrate is concerned. Likewise, the dissociation rate constant will not decrease further with increasing chain length. Although an increase in substrate chain length above the dimensions of the peptide binding site may lead to a proportional increase in k_a, when the interaction radius of the substrate is increased, this proportional increase is the upper limit, since the effect of the increased interaction radius will be at least partially neutralized by the adverse effect of the chain length on the diffusion rate of the substrate. From the values for k_{cat} (4 s^{-1}) and K_m (2.5×10^{-6} M) for $(Pro\text{-}Pro\text{-}Gly)_{20}$ (Kivirikko & Myllylä, 1980), the lower limit for the value of k_a can be calculated, being at least 1.6×10^6 M^{-1} s^{-1}, by assuming that the formation of an enzyme–substrate complex leads to hydroxylation of one proline residue in the substrate. The value of k_a for procollagen (4 s^{-1} divided by 2×10^{-9} M) would be at least 2×10^9 M^{-1} s^{-1}. The ratio of association rate constants between procollagen and $(Pro\text{-}Pro\text{-}Gly)_{20}$ would be 1.25×10^3, this value being much higher than the ratio predicted from the difference in chain length, which is only 17-fold. The remaining increase in association rate constant over almost 2 orders of magnitude cannot readily be explained by differences in amino acid composition or electrostatic interactions, as these effects, when studied on the K_m values of synthetic peptides, were far less pronounced (Kivirikko & Myllylä, 1980). Evidently, some mechanism must exist to increase the association rate for substrate sites on a large peptide by removing diffusional constraints on the rate of collision of enzyme and substrate.

We propose here a model for such a mechanism based on the fact that the enzyme acts as a dimer. The enzyme consists of two 64-kDa α and two 60-kDa β subunits (Kivirikko & Myllylä, 1980). There are two ferrous iron atoms present per $\alpha_2\beta_2$ (de Jong & Kemp, 1982), and each one of the two α subunits bears a peptide binding site (de Waal et al., 1985). Our hypothesis is that the ability of the enzyme to bind simultaneously to two peptide substrates is used to bring about transient doubly bound complexes of the enzyme with two domains of a large peptide with multiple substrate sites, facilitated by thermal fluctuations within the peptide. At the moment that the one binding site dissociates from a newly hydroxylated peptidyl proline, the other site is already or still bound to another peptidyl proline on the same substrate molecule, thereby preventing rapid dissociations of the enzyme–substrate complex between successive hydroxylations. This allows the enzyme to hydroxylate a number of peptidyl prolines at a high rate (approximating the k_{cat}) on the same substrate molecule, leading to a low K_m by overcoming the diffusional limitations on the association rate, the search for hydroxylatable proline residues in the substrate taking place in a reduced volume and dimensionality [reviewed by Berg and Von Hippel (1985)].

In the mechanism outlined above, there is a synergistic relation between the two peptide binding sites of prolyl 4-hydroxylase in the sense that two binding sites on one enzyme molecule are far more efficient in hydroxylation of a large peptide substrate at a nonsaturating concentration than two separate binding sites on two enzyme molecules would be. We prefer to use the term processive model in order to distinguish the mechanism proposed here from the common mechanism of positive cooperativity, in which an event, such as binding of a substrate or activator, actually enhances the binding properties of the other binding site(s).

If the proposed model is correct, a synergistic relation is not to be found in the hydroxylation of a short peptide substrate, where simultaneous binding of two moieties of the substrate at both the active sites on the same enzyme molecule cannot take place. We tested the model and predictions by photoaffinity labeling of the enzyme with *N*-(4-azido-2-nitrophenyl)glycyl-$(Pro\text{-}Pro\text{-}Gly)_5$. This peptide photoaffinity label prevents the binding of the peptide substrate to the catalytic center, while leaving the catalytic center intact with respect to the other substrates (de Waal et al., 1985). Enzyme molecules photoaffinity-labeled at one binding site are predicted to act as monomeric, nonprocessive enzymes, consequently, with a high K_m (see Appendix II) of hydroxylation of a large peptide substrate. The photoaffinity label 5-azidopyridine-2-carboxylic acid (de Waal et al., 1987), developed to fit into the 2-oxoglutarate binding site that was extensively characterized by Majamaa et al. (1984) using several pyridine carboxylates, is used as a means to inhibit the enzyme without disturbing the peptide binding site. Enzyme molecules with one binding site photoaffinity labeled are in this case predicted to retain processive properties in the hydroxylation of a large peptide substrate.

EXPERIMENTAL PROCEDURES

$(Pro\text{-}Pro\text{-}Gly)_5 \cdot 5H_2O$ was obtained from Protein Research Foundation (Minoh-shi, Osaka, Japan).

Protein concentration was determined according to the method of Peterson (1977).

Isolation of prolyl 4-hydroxylase, synthesis of *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$, and determination of radioactivity on subunits of photoaffinity-labeled enzyme were performed as described (de Waal et al., 1985). The specific radioactivity of *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$ was 20.6 dpm/pmol.

5-Azidopyridine-2-carboxylic acid was synthesized as described (de Waal et al., 1987).

Prolyl 4-hydroxylase was photoaffinity labeled with *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ by irradiation at 366 nm with a Camag UV lamp. The concentration of photoaffinity label was 0.1 or 0.2 mM. Irradiation time varied between 2 and 15 min. The irradiated mixture was cooled on ice–water in a siliconized glass vessel. The enzyme was separated from photolyzed photoaffinity label by centrifugation through Sephadex G-50 according to the method of Penefsky (1977). The procedure was repeated up to 6 times. The same procedures with (Pro-Pro-Gly)$_5$ instead of photoaffinity label were carried out as a control to allow for correction of nonspecific inactivation, typically less than 20% after five irradiations. When radiolabeled photoaffinity label was used, the noncovalently attached label remaining after the Penefsky columns was removed by equilibrium gel filtration on a Pharmacia Superose 12 FPLC column.

Prolyl 4-hydroxylase was alternatively photoaffinity labeled with 5-azidopyridine-2-carboxylic acid by UV irradiation with a Pen-Ray UV lamp (UV Products Inc., San Gabriel, CA) without filter, positioned 2 mm above at 20-μL glass capillary cooled with ice–water. The 5-azidopyridine-2-carboxylic acid concentration was 1 mM. The irradiation time was 5 min. The enzyme was separated from photolyzed photoaffinity label by centrifugation through Sephadex G-50. The procedure was repeated once or twice. The same procedures with pyridine-2-carboxylic acid instead of photoaffinity label were carried out as a control.

The irradiated enzyme mixtures were made up in buffer containing 0.2 M glycine, 0.2 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.8, 4 °C), and 10^{-5} M dithiothreitol. The enzyme was diluted in the same buffer with 10^{-4} M dithiothreitol and 1 mg/mL bovine serum albumin.

Procollagen was isolated according to the method of Kivirikko and Myllylä (1982).

Activity of prolyl 4-hydroxylase was measured as [^{3}H]H_2O release from [4-^{3}H]proline-labeled procollagen as described by Peterkofsky and DiBlasio (1975) or as [^{14}C]CO_2 release from 2-oxo-[1-^{14}C]glutarate as described before (de Jong & Kemp, 1984) with (Pro-Pro-Gly)$_5$ as substrate. Final concentrations during the reactions were 0.05 M Tris-HCl (pH 7.7 at 20 °C), 0.1 mg/mL catalase, 2 mg/mL bovine serum albumin, 0.2 mM dithiothreitol, 1 mM ascorbate, 10^{-5} M $FeSO_4$, 10^{-4} M 2-oxoglutarate, and 0.5 mM (Pro-Pro-Gly)$_5$ or procollagen at the K_m. The final volume of the reaction medium was 0.5 mL with the synthetic substrate and 0.1 mL with the procollagen. The reaction temperature was 37 °C except for the experiment in Figure 2 (30 °C).

Results

The loss of enzyme activity with (Pro-Pro-Gly)$_5$ as a substrate upon photoaffinity labeling with *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$ is linearly related to the amount of photoaffinity label covalently bound to the enzyme, as is shown in Figure 1. The line extrapolates to 100% inhibition at 2.4 mol of label/mol of enzyme. This reflects, within experimental error, the binding of 2 mol of label/mol of enzyme, as was consistently observed in previous experiments (de Waal et al., 1985).

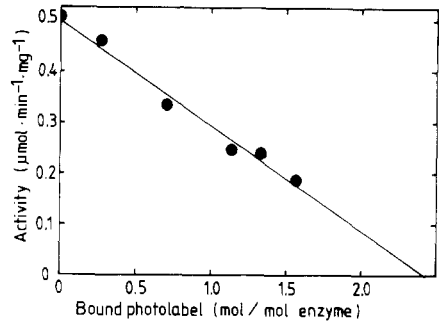

FIGURE 1: Relation between amount of bound photoaffinity label and remaining enzyme activity. Prolyl 4-hydroxylase was irradiated up to 5 times with *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$ as described under Experimental Procedures. The control was irradiated 5 times in the presence of (Pro-Pro-Gly)$_5$. Enzyme activity was determined with (Pro-Pro-Gly)$_5$ as substrate. The line through the experimental points was calculated by geometric mean regression analysis (Sokal & Rohlf, 1981). Enzyme activity was determined in duplicate.

The linear behavior shown in Figure 1 is consistent with the assumptions that the binding properties of the two peptide binding sites of prolyl 4-hydroxylase are independent of each other (in other words, the chance for a binding site to be photoaffinity labeled is not affected by labeling of the other site on the enzyme) and that the activity of the nonlabeled site of enzyme molecules with one peptide binding site occupied with photoaffinity label is not affected (see Appendix I).

Figure 2 reveals that the second assumption cannot be valid for hydroxylation of a long peptide substrate. Here the activity of the same batch of photoaffinity-labeled enzyme (bearing 1.57 mol of label/mol of enzyme) was measured with (Pro-Pro-Gly)$_5$ (panel A) and with procollagen (panel B) as substrate. No difference in K_m for (Pro-Pro-Gly)$_5$ was observed between the enzymes irradiated with and without photoaffinity label. The remaining activity with (Pro-Pro-Gly)$_5$ was 36.5%. From this value it was calculated that the fractions of enzyme with zero, one, and two sites occupied by the photoaffinity label were 0.133, 0.464, and 0.403, respectively (see Appendix I). The points in the Lineweaver–Burk plot with procollagen as a substrate (Figure 2B, triangles) do not fit with a simulation (dashed line) that is based on the assumption that the fraction of enzyme with only one free site would have the same K_m toward the procollagen substrate as nonlabeled enzyme. In contrast, the simulated line based on the assumption that this fraction has a 5-fold higher K_m (drawn line) fits well with the experimental points (see Appendix I). This result is in agreement with the prediction of our processive model. It should be noted that our determination of the K_m for procollagen is only relative, since we do not know the specific radioactivity of the substrate. With assumptions concerning the k_{cat} and number of hydroxylatable proline residues, we calculated that the K_m is in the range of 1–10 nM with several batches of procollagen and therefore not different from published results (Berg & Prockop, 1973).

The substrate-type dependent difference in activity remaining upon photoaffinity labeling was confirmed by a series of enzyme preparations with varying extents of inhibition. The remaining activity was now measured only at the K_m of peptide substrate. Figure 3 is a two-dimensional scatter diagram with percentages of remaining activity when procollagen and (Pro-Pro-Gly)$_5$ are used as substrates. When the activity is independent of the type of substrate, the data should fit a straight line connecting 0 and 100% activity. This was the case for the enzyme photoaffinity labeled with the 2-oxo-

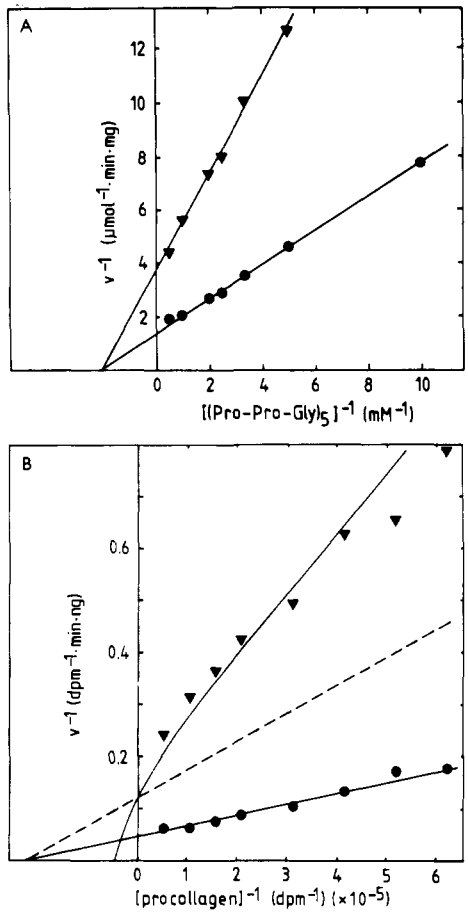

FIGURE 2: Lineweaver–Burk plots with $(Pro\text{-}Pro\text{-}Gly)_5$ (panel A) or procollagen (panel B) as varied substrates. Prolyl 4-hydroxylase was irradiated 5 times in the presence of *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-$(Pro\text{-}Pro\text{-}Gly)_5$ (triangles) or $(Pro\text{-}Pro\text{-}Gly)_5$ (circles) as described under Experimental Procedures. All data points were determined in duplicate. The line drawn through the experimental points (triangles) in panel B is a simulation based on the assumption that enzyme molecules with only one free active site have a 5-fold higher K_m than nonlabeled enzyme molecules. The dashed line is a simulation based on the assumption that the K_m values for nonlabeled enzyme and enzyme with one free site are equal.

glutarate analogue photoaffinity label (triangles) but clearly not for the enzyme photoaffinity labeled with the peptide photoaffinity label (circles). The activities of enzyme photoaffinity labeled with the peptide photoaffinity label fit much better to the parabola in Figure 3, which is a simulation of the relation between enzyme activities for a short and a long substrate, assuming that enzyme molecules with one peptide binding site have, in this case, a 10 times higher K_m with the long substrate than nonlabeled enzyme (see Appendix I). This result therefore confirms the model in which the two peptide binding sites of prolyl 4-hydroxylase act together on long peptide substrates to increase hydroxylation efficiency.

DISCUSSION

The basic feature of the processive model proposed here is that after an association with a multisubstrate complex the enzyme remains bound to it for a long time. Within this time the search for hydroxylatable sequences in the substrate takes place in a reduced volume and dimensionality, thus facilitating the finding of substrate sites in a way than can overcome the diffusional limits of association rates (Berg & Von Hippel, 1985). Such a mechanism explains the large difference in K_m that is observed between peptide substrates of different chain length (Appendix II). The mechanism may involve interdomain transfer of the enzyme via doubly bound intermediate

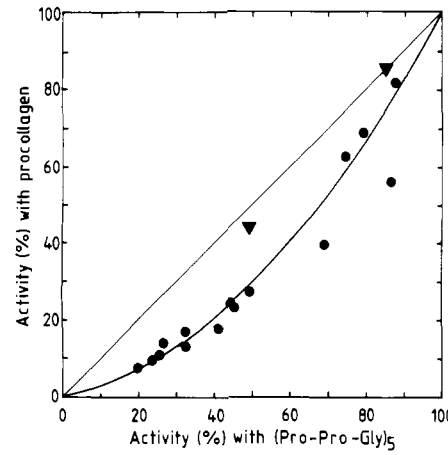

FIGURE 3: Remaining enzyme activity after photoaffinity labeling measured with a short (abscissa) and a long (ordinate) peptide substrate at their respective K_m. Prolyl 4-hydroxylase was irradiated up to 6 times in the presence of *N*-(4-azido-2-nitrophenyl)glycyl-$(Pro\text{-}Pro\text{-}Gly)_5$ (circles) or up to 2 times in the presence of 5-azidopyridine-2-carboxylic acid (triangles) as described under Experimental Procedures. For each condition the activity of the control was taken as 100%. All data points were determined in triplicate. The drawn lines are simulations with the assumption that enzyme molecules with one site occupied by photoaffinity label have half the activity and the same K_m as nonlabeled enzyme with respect to both $(Pro\text{-}Pro\text{-}Gly)_5$ and procollagen (straight line) or have a K_m for procollagen that is 10 times higher than the K_m for procollagen of nonlabeled enzyme (parabola).

states, sliding of the enzyme between strands of the substrate, rolling of the enzyme along one strand, or a combination of these mechanisms. Similar models have been proposed for the association of the *Escherichia coli lac* repressor with *lac* operator-containing DNA (Winter et al., 1981; Fried & Crothers, 1984). The possibility of mechanisms allowing for several successive hydroxylations by prolyl 4-hydroxylase within the same prollagen substrate molecule has also been suggested before (Juva & Prockop, 1969; Prockop & Kivirikko, 1969; Prockop et al., 1976), but no experimental evidence could be obtained.

The mechanism proposed here is completely dependent on the presence of more than one substrate binding site. When one site of two is blocked by a photoaffinity label, the enzyme will dissociate from the substrate after each hydroxylation, leading to a renewed search for the next hydroxylatable peptidyl proline in three dimensions. The result is a much higher K_m for a long substrate molecule such as procollagen (see Appendix II). The K_m for a small peptide substrate such as $(Pro\text{-}Pro\text{-}Gly)_5$ is not likely to be affected by the loss of one of two binding sites because the diameter of the enzyme exceeds the length of the substrate, making binding of the same peptide at both binding sites of one enzyme impossible. Experimentally, we found that the enzyme hydroxylates the short peptide with the same K_m with one peptide binding site as it does with two (Figure 2A). We demonstrated that the low K_m of procollagen is dependent on two binding sites (Figure 2B). We also compared the activities with a long and a short substrate of enzyme photoaffinity labeled, to a varying extent, with *N*-(4-azido-2-nitrophenyl)glycyl-$(Pro\text{-}Pro\text{-}Gly)_5$. These results (Figure 3) are likewise in agreement with the assumption that enzyme molecules with one free peptide binding site have a much higher K_m for the long substrate than enzyme molecules with two peptide binding sites.

The difference in K_m values between nonlabeled enzyme and enzyme with only one free peptide binding site suggests that 5–10 proline residues may be hydroxylated in one encounter between enzyme and procollagen (see Appendix II). However,

this figure may represent the lower limit, since it cannot be excluded that enzyme molecules labeled on one subunit with *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ still retain some processive properties, brought about by binding subsites that are not blocked by the covalently bound peptide photolabel. Our previous interpretation (de Waal et al., 1985) that the binding subsites are discrete binding sites capable of binding up to 8 mol of photolyzed noncovalently bound *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$/mol of enzyme turned out to be erroneous, but the ability of enzyme photoaffinity labeled at two sites to bind to poly(L-proline) (de Waal et al., 1985) clearly indicates that additional binding sites must exist. This binding capacity not located in the catalytic center is more likely to be weak and diffuse and, therefore, well suited to bring about initial contact with a substrate molecule without forming stable, nonproductive complexes. In addition, enzyme–substrate complexes can be stabilized by the binding subsites.

The processive mechanism explains why the enzyme should possess two active sites. Why there are two types of monomer is still an unsolved question. Although the binding sites for 2-oxoglutarate (de Waal et al., 1987; Günzler et al., 1987) and the peptide substrate (de Waal et al., 1985) are shown to be located on the α subunit, a function of the β subunit in the catalysis of hydroxylation cannot be excluded in light of the existence of an activity-inhibiting monoclonal antibody against the β subunit (Höythyä et al., 1984) and ascorbate-sensitive affinity label incorporation into α and β subunits (Günzler et al., unpublished results). The recent discoveries that the β subunit is the product of the same gene as that of the enzyme protein disulfide isomerase (Pihlajaniemi et al., 1987) and has indeed the corresponding activity when incorporated in the prolyl 4-hydroxylase tetramer (Koivu et al., 1987) suggest that the prolyl 4-hydroxylase is actually an enzyme complex. It is tempting to speculate that the processive action in the hydroxylation in vivo is accomplished between three growing pro-α strands, which after completion of hydroxylation and protein synthesis are disulfide-linked by the hydroxylating enzyme.

We describe here the processive model only for prolyl 4-hydroxylase. It is very likely that prolyl 3-hydroxylase and lysyl hydroxylase act according to the same mechanism. The mechanism has, to our knowledge, not been described for any protein–protein interaction but may well be of importance for other enzymes that act on polymeric substrates.

Acknowledgments

We thank Dr. J. A. Berden, Prof. Dr. K. Van Dam, and Prof. Dr. A. J. B. M. Van Renswoude for helpful discussions and reading the manuscript.

Appendix I

Enzyme Activity with a Short and a Long Polymeric Substrate after Photoaffinity Labeling of an Enzyme Containing Two Equal and Independent Binding Sites. For an enzyme with two equal and independent binding sites for a photoaffinity label, the partitioning of x ($0 \leq x \leq 2$) mol of label/mol of enzyme will be

$$f_0 = (1 - x/2)^2 \quad (1)$$

$$f_1 = (1 - x/2)x \quad (2)$$

$$f_2 = (x/2)^2 \quad (3)$$

where f_0, f_1, and f_2 denote the fraction of enzyme molecules with 0, 1, and 2 sites occupied by the photoaffinity label, respectively.

The fractional activity p of photoaffinity-labeled enzyme is given by

$$p = v_x/v \quad (4)$$

where v and v_x are the enzyme activities of nonlabeled enzyme and of enzyme labeled with x mol of photolabel/mol of enzyme, respectively.

Only nonlabeled enzyme molecules and enzyme molecules with still one free active site contribute to the activity. Therefore

$$v_x = f_0 v + f_1 v^* \quad (5)$$

where v^* is the activity of enzyme with one binding site photoaffinity labeled. Substituting

$$v = VS/(S + K_m) \quad (6)$$

$$v^* = V^*S/(S + K_m^*) \quad (7)$$

and

$$V^* = V/2 \quad (8)$$

into eq 4 and 5 gives, after some rearrangements

$$p = f_0 + \frac{f_1}{2}\frac{S + K_m}{S + K_m^*} \quad (9)$$

where K_m is the K_m of a substrate with nonlabeled enzyme and K_m^* is the K_m of this substrate with an enzyme fraction in which one of the two binding sites is blocked by a photoaffinity label. Substitution of eq 1 and 2 into eq 9 gives

$$p = \left(1 - \frac{x}{2}\right)^2 + \frac{x}{2}\left(1 - \frac{x}{2}\right)\frac{S + K_m}{S + K_m^*} \quad (10)$$

or

$$p = \frac{x^2}{4}\left(1 - \frac{S + K_m}{S + K_m^*}\right) + \frac{x}{2}\left(\frac{S + K_m}{S + K_m^*} - 2\right) + 1 \quad (11)$$

From eq 11 it follows that the fractional activity q for the case that $K_m = K_m^*$ is given by

$$q = 1 - (x/2) \quad (12)$$

The fractional activity p with a long substrate with $K_m^* > K_m$ can be expressed as a function of the fractional activity q with a short substrate obeying eq 12:

$$p = q^2\left(1 - \frac{S + K_m}{S + K_m^*}\right) + q\left(\frac{S + K_m}{S + K_m^*}\right) \quad (13)$$

In the special case where S equals K_m, i.e., the conditions used to obtain the results shown in Figure 3, eq 13 can be simplified to

$$p = q^2\left(1 - \frac{2K_m}{K_m + K_m^*}\right) + q\left(\frac{2K_m}{K_m + K_m^*}\right) \quad (14)$$

This equation was used for the simulation as shown in Figure 3. The equation for the simulated lines in Figure 2B was obtained by combining eq 5–8.

Appendix II

Comparison between a Processive and a Nonprocessive Mechanism with Respect to the K_m of the Substrate. The processive mechanism will allow for n successive catalytic events without intermediate dissociations of the enzyme–substrate complex. In the case where the time needed for rearrangements within the enzyme–substrate complex is negligible, we can write

$$t_n = (n/k_{cat}) + t_a \quad (15)$$

where t_n stands for the time needed for n catalytic events and

t_a for the time between dissociation of the enzyme–substrate complex and reassociation. Since the turnover rate v equals by definition n/t_n, we obtain from eq 15

$$v = k_{cat}n/(n + t_a k_{cat}) \quad (16)$$

According to Michaelis–Menten kinetics we can write

$$v = k_{cat}S/(S + K_m^n) \quad (17)$$

where K_m^n stands for the K_m of a substrate that is n times modified before dissociation of the enzyme–substrate complex. Combining eq 16 and 17, we have

$$t_a = K_m^n n/Sk_{cat} \quad (18)$$

In the special case of a dimeric enzyme that is photoaffinity labeled at one site, n will be 1, since the enzyme–substrate complex will now dissociate after each catalytic event. In this case k_{cat} will be half that of the nonlabeled enzyme. Likewise, the time between dissociation and reassociation will now be twice as much as that of the nonlabeled enzyme, since only one of the two active sites is free. It is realized that after the first modification of, and release from, a polymeric substrate a higher probability may exist for a subsequent collision with the same substrate molecule, and consequently for a second modification, than for a collision with another substrate molecule, at least at a low substrate concentration. Consequently, the time between dissociation and reassociation will be less, actually, than $2t_a$. However, this is not further taken into account below. Therefore, we obtain in this case, analogous to the derivation of eq 18

$$t_a = K_m^1/Sk_{cat} \quad (19)$$

From 18 and 19 we obtain

$$K_m^1 = K_m^n n \quad (20)$$

In other words, the 10-fold difference in K_m values of photoaffinity-labeled and nonlabeled enzyme for procollagen (cf. Figure 3 and Appendix I) reflects the successive hydroxylations of at least 10 proline residues in procollagen molecules.

From eq 18 we can also derive the relation between K_m values of a long substrate that is modified n times before dissociation of the enzyme–substrate complex and of a short substrate occupying all binding subsites that is not able to interact with two catalytic binding sites and, consequently, is modified only once. Now t_a depends on the substrate. If we assume that the effect of the decreased diffusion rate of the long substrate on t_a is balanced by the effect of the increased interaction radius, eq 20 provides a rough estimation of the effect of n on the K_m of polymeric substrates in a processive mechanism.

Registry No. (Pro-Pro-Gly)$_5$, 24030-52-2; *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$, 98652-64-3; prolyl 4-hydroxylase, 9028-06-2.

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