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Photoaffinity Labeling of Peptide Binding Sites of Prolyl 4-Hydroxylase with *N*-(4-Azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$†

Anthony de Waal, Luitzen de Jong,* Aloysius F. Hartog, and Albertus Kemp‡
Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, 1000 HD Amsterdam, The Netherlands
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ABSTRACT: The synthesis is described of the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ for the peptide binding site of prolyl 4-hydroxylase. The photoaffinity label is a good substrate and is capable of light-induced inactivation of prolyl 4-hydroxylase activity. Inactivation depends on the concentration of photoaffinity label and is prevented by competition with excess (Pro-Pro-Gly)$_5$. Two moles of photoaffinity label per mole of enzyme is needed for 100% inactivation of enzymic activity. Oxidative decarboxylation of 2-oxoglutarate measured in the absence of added peptide substrate is not affected by labeling. We conclude that the covalently bound nitreno derivative of *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ acts by preventing the binding of peptide substrate to the catalytic site without interfering with the binding of the other substrates and cofactors 2-oxoglutarate, O_2, Fe^{2+}, and ascorbate. Labeling is specific for the α subunit of the tetrameric $\alpha_2\beta_2$ enzyme. In addition to two catalytic binding sites that are blocked by the photoaffinity label, the enzyme contains binding subsites for peptide substrates, as judged from the capability of photoinactivated enzyme to bind to a poly(L-proline) affinity column. These binding subsites may account for the rapidly increasing affinity for peptide substrates with increasing chain length.

Prolyl 4-hydroxylase [prolyl-glycyl-peptide, 2-oxoglutarate: oxygen oxidoreductase (4-hydroxylating), EC 1.14.11.2] catalyzes the posttranslational modification of certain peptidyl proline residues in nascent pro-α chains of procollagen. This modification results, with slight differences in the various types of collagen, in the formation of approximately 100 hydroxyproline residues per pro-α chain. Hydroxylation of proline residues is essential for the thermal stability of the triple helical structure of the end product collagen (Berg & Prockop, 1973).

The enzyme has as substrates 2-oxoglutarate, molecular oxygen, and a peptide containing one or more -X-Pro-Gly- sequences (X denoting any amino acid but glycine), and it requires ferrous ions and ascorbate. One atom of the oxygen is used for the oxidative decarboxylation of 2-oxoglutarate to succinate (Rhoads & Udenfriend, 1968). The incorporation of the other atom into proline results in the formation of *trans*-4-hydroxyproline. The hydroxylation reaction is most likely via a ferryl complex as the oxygen-transferring intermediate (Siegel, 1979; Hanauske-Abel & Günzler, 1982; de Jong & Kemp, 1984). The reaction sequence resulting in hydroxylation of proline does not require the reducing agent ascorbate. However, in addition to the complete reaction in

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* Address correspondence to this author c/o Ms. G. E. E. van Noppen, F.I.L., Publications Secretary.
‡ Deceased Feb 12, 1985.

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the presence of a peptide substrate, the enzyme is able to catalyze the oxidative decarboxylation of 2-oxoglutarate without concomitant hydroxylation of peptidyl proline (Tuderman et al., 1977; Counts et al., 1978; Rao & Adams, 1978). It is in these uncoupled reaction cycles, which may even occasionally occur as a side reaction in the presence of a substrate capable of being hydroxylated, that ascorbate is consumed stoichiometrically with 2-oxoglutarate and O_2 (de Jong & Kemp, 1984; Myllylä et al., 1984). Ascorbate consumption prevents inactivation of the enzyme by keeping the two iron atoms bound per tetrameric $\alpha_2\beta_2$ enzyme molecule (de Jong & Kemp, 1982) in the ferrous state, as has been shown by ESR[1] spectroscopy (de Jong et al., 1982).

The minimal requirement for a peptidyl proline substrate to be hydroxylated seems to be fulfilled by the tripeptide X-Pro-Gly, provided that its carboxy terminus is blocked with *N*-methylamide. However, there is a strikingly increasing affinity for peptide substrates with increasing chain length [for a review, see Kivirikko & Myllylä (1980)]. On the basis of the preferential hydroxylation of the penultimate triplets from the carboxy terminus in the synthetic polypeptides (Pro-Pro-Gly)$_5$ (Kivirikko et al., 1971) and (Pro-Pro-Gly)$_{10}$ (Berg et al., 1977), it has been suggested that prolyl 4-hydroxylase has an asymmetric active site in which binding subsites are located adjacent to the catalytic subsite (Berg et al., 1977). At the catalytic site, the peptide may adopt a β-turn conformation (Chopra & Ananthanarayanan, 1982).

In order to investigate the structure of the peptide binding site and its environment including the catalytic site, we decided to develop photoaffinity labels. We describe here the synthesis of the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ and our first experimental results.

Experimental Procedures

Materials. (Pro-Pro-Gly)$_5$·5H$_2$O was obtained from Protein Research Foundation (Minoh-shi, Osaka, Japan). [2-^{3}H]-Glycine and 2-oxo[1-^{14}C]glutarate were obtained from Amersham International plc. (Amersham, Buckinghamshire, U.K.). *N*-Hydroxysuccinimide and 4-fluoro-3-nitrophenylazide were obtained from Pierce Chemical Company (Rockford, Il). Dicyclohexylcarbodiimide was obtained from Janssen Chimica (Beerse, Belgium). Poly(L-proline) type II, approximate M_r 8000, was obtained from Sigma Chemical Co. (St. Louis, MO). Protosol was obtained from New England Nuclear (Boston, MA).

Synthesis of the Photoaffinity Label N-(4-Azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ [NAG-(Pro-Pro-Gly)$_5$]. All procedures were carried out in the dark. *N*-(4-Azido-2-nitrophenyl)glycine (NAG) and its *N*-hydroxysuccinimide ester (NAG-NOS) were synthesized according to methods developed by Fleet et al. (1972) and Hsiung & Cantor (1974) as described by Ofengand et al. (1977), with [2-^{3}H]glycine to introduce radioactive label. The same procedure was carried out with nonradioactive glycine. The reaction mixture from the NAG-NOS synthesis, containing 25 μmol of NAG-NOS in 2 mL of water-free dimethoxyethyl ether, was added to a solution of 10 μmol of (Pro-Pro-Gly)$_5$ and 100 μmol of triethylamine in 0.5 mL of water. The mixture (pH 7.5) was stirred overnight at 20 °C and then dried in vacuo. The dry material was dissolved in 3 mL of water and extracted 5 times with 3 mL of butanol to remove free NAG and NAG-NOS. After evaporation of butanol and concentration to 2 mL in vacuo, the solution was brought onto a DEAE-Sephadex A-25 column equilibrated in water (pH 6). The column (20 × 0.5 cm) was washed with 75 mL of water to remove free (Pro-Pro-Gly)$_5$, NOS, and remaining NAG-NOS. NAG-(Pro-Pro-Gly)$_5$ was eluted from the column with 50 mM triethylamine–HCO$_3$ (pH 7.8), yielding one peak of absorption at 220 nm coinciding with one peak of radioactivity. The peak fraction was dried in vacuo at 15 °C and coevaporated with 100% ethanol. The product, isolated as triethylamine salt, was dissolved in ethanol–water (1:1) at a concentration of 3 mM at pH 5 and stored at −20 °C. Under these conditions, the product was found to be stable for at least 16 months as judged from its absorption spectrum. The concentration was estimated with millimolar extinction coefficients of 23.0 at 260 nm and 5.0 at 460 nm (Chicheportiche et al., 1979). The specific radioactivity calculated from these values was 77 dpm/pmol, with a yield of 5% of the starting amount of glycine. The product was analyzed by silica gel thin-layer chromatography on Merck 60 F_{254} gel plates with ethyl acetate, butanol–acetic acid–water (5:2:3), and ethanol–NH$_4$OH–water (10:1:10), respectively, as eluents. R_f values for each substance (R_f in ethyl acetate/R_f in butanol–acetic acid–water/R_f in ethanol–NH$_4$OH–water) are as follows: NAG-(Pro-Pro-Gly)$_5$, 0.00/0.03/0.86; (Pro-Pro-Gly)$_5$, 0.00/0.10/0.65; NAG, 0.10/0.67/0.90; NAG-NOS, 0.85/0.80/0.94; NOS, 0.50/0.12/0.78; 4-fluoro-3-nitrophenylazide, 0.92/0.85/0.98. Silica gel on spots corresponding to these R_f values was scratched off, mixed with 0.5 mL of water for 1 h, and counted in 4 mL of a scintillation cocktail. Radioactivity was solely found on NAG-(Pro-Pro-Gly)$_5$ spots. The isoelectric point was 3.5. The absorption spectrum is given in Figure 1.

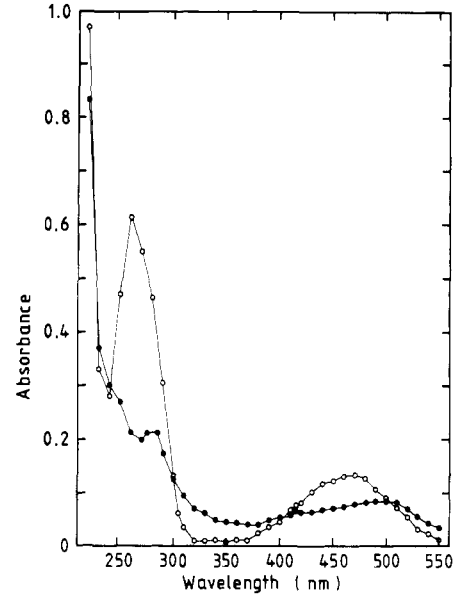

FIGURE 1: Absorption spectrum of the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$ before and after photolysis. The photoaffinity label was dissolved in water at a concentration of 15 μM, and absorbance measurements were carried out manually on a Zeiss spectrophotometer before (open symbols) and after (closed symbols) illumination with 366 nm at 0 °C at a distance of 4 cm from a Camag de luxe ultraviolet lamp for 20 min.

Photoinhibition of Prolyl 4-Hydroxylase. Photolysis of NAG-(Pro-Pro-Gly)$_5$ was done by illumination at 366 nm, with a Camag ultraviolet lamp. Samples containing 10 μM

[1] Abbreviations: NOS, *N*-hydroxysuccinimide; NAG, *N*-(4-azido-2-nitrophenyl)glycine; NAG-NOS, *N*-hydroxysuccinimide ester of *N*-(4-azido-2-nitrophenyl)glycine; NAG-(Pro-Pro-Gly)$_5$, *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$; NaDodSO$_4$, sodium dodecyl sulfate; ESR, electron spin resonance; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; DEAE, diethylaminoethyl; LB, low ionic strength buffer containing 0.05 M glycine, 0.05 M NaCl, 0.05 M Tris-HCl (pH 7.4, 4 °C), and 10^{-5} M dithiothreitol.

prolyl 4-hydroxylase, 210 μM NAG-(Pro-Pro-Gly)$_5$, 0.2 M glycine, 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.8, 4 °C), and 10^{-5} M dithiothreitol, in a final volume of maximally 100 μL, were illuminated at a distance of 4 cm for 15 min in siliconized glass vessels cooled in melting ice. Under these conditions, all NAG-(Pro-Pro-Gly)$_5$ was photolyzed. After illumination, enzyme and photolabel were separated by centrifugation through gel filtration columns according to Penefsky (1977). The column consisted of Sephadex G-50 fine, equilibrated in 0.2 M glycine, 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.8, 4 °C), and 10^{-5} M dithiothreitol. When desired, NAG-(Pro-Pro-Gly)$_5$ was added to the eluate, containing the enzyme, for another round of illumination. Enzyme was illuminated up to 5 times. After the last illumination, the enzyme was treated twice by the Penefsky procedure. However, such enzyme preparations contained, in addition to covalently bound photolabel, significant amounts of noncovalently bound photolyzed NAG-(Pro-Pro-Gly)$_5$ (up to 8 mol/mol of enzyme). Therefore, in some experiments, the enzyme was further purified by equilibrium gel filtration on Sepharose 6B. A 40 cm long column, with a bed volume of 35 mL, was equilibrated in a solution containing 0.2 M glycine, 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.8, 4 °C), and 10^{-5} M dithiothreitol. The enzyme was eluted at a flow rate of 2–3 mL/h. Fractions were pooled and concentrated by ultrafiltration. The concentrates were centrifuged at 11000*g* for 5 min, at 4 °C. Supernatants were stored in liquid nitrogen for further use.

Determination of Radioactivity on Subunits. Prolyl 4-hydroxylase subunits were separated by polyacrylamide disc gel electrophoresis in the presence of $NaDodSO_4$ according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue and scanned at 500 nm on a Zeiss spectrophotometer with gel-scanning attachment and scale expander. Gels were sliced to 1-mm slices with a Mickle gel slicer. Slices were incubated overnight in Protosol with 10% water. The solution was neutralized before addition of 4 mL of Packard Scintillator 299 scintillation cocktail. Counting vials were kept in the dark for 4 h before counting in the tritium channel of a Packard Tri-Carb liquid scintillation counter.

Activity Measurement. Determination of prolyl 4-hydroxylase activity was done polarographically, following O_2 consumption with a Clarke-type electrode (Nietfeld & Kemp, 1980), in a final volume of 0.21 mL, or by measuring $^{14}CO_2$ release from 2-oxo[1-^{14}C]glutarate as described (de Jong & Kemp, 1984). Final concentrations during the reactions were 50 mM 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 $FeSO_4$, 0.1 mM 2-oxoglutarate, and, unless otherwise stated, 0.455 mM (Pro-Pro-Gly)$_5$. When NAG-(Pro-Pro-Gly)$_5$ was used as substrate, measurements were made in the dark.

Protein. Protein determination was according to the method of Lowry et al. (1951) as modified by Peterson (1977).

Enzyme Isolation. The isolation of prolyl 4-hydroxylase was done with a modification of the procedures reported by Tuderman et al. (1975) and Kedersha & Berg (1981). One hundred chick embryos, 17 days old, with a total weight of about 3 kg, were mixed with 3 L of homogenization buffer, consisting of 0.1 M glycine, 0.1 M NaCl, 0.1 wt % Triton X-100, and 0.01 M Tris-HCl (pH 7.8, 4 °C). Homogenization was carried out in a precooled CB-6 Waring Commercial Blendor for 1 min at 12000 rpm and twice for 1 min at 15000 rpm. The homogenate was centrifuged for 1 h, 20000*g*, at 4 °C. The supernatant was filtered over a double layer of cheesecloth to remove fat and quickly brought to room temperature in a water bath. The pooled filtrate was mixed with Sepharose 4B having poly(L-proline) (M_r 40000) covalently attached. The bed volume of this affinity material was 300 mL. The homogenate was shaken with the affinity material for 1 h and quickly cooled in ice–water. Affinity material was separated from the homogenate by centrifugation for 6 min, 1000*g*, 4 °C, in an MSE Mistral 6L. The collected affinity material was taken up in 1 L of wash buffer containing 0.1 M glycine, 0.1 M NaCl, and 0.01 M Tris-HCl (pH 7.8, 4 °C) and centrifuged again. The affinity material was poured onto a glass filter and washed with 8 L of wash buffer. The affinity material was poured into a column with a diameter of 4 cm and washed with 1 volume of low ionic strength buffer (LB) consisting of 0.05 M glycine, 0.05 M NaCl, 0.05 M Tris-HCl (pH 7.4, 4 °C), and 10^{-5} M dithiothreitol. The enzyme was eluted from the column with 170 mL of LB, containing 3 mg of poly(L-proline) (M_r 8000) per mL. The eluate was directly passed through a column of DEAE-cellulose, with a diameter of 1.5 cm and a bed volume of 40 mL, at a flow rate of 70 mL h^{-1} cm^{-2}. The DEAE-cellulose was washed overnight with approximately 1 L of LB. Enzyme was eluted from the column with a linear gradient of NaCl in LB from 50 to 350 mM NaCl, at a flow rate of 17 mL h^{-1} cm^{-2}. Peak fractions with a total volume of 100 mL were concentrated to a final volume of 4 mL in an Amicon ultrafiltration cell with a PM30 filter at a pressure of 1.5–2 bar. The concentrated enzyme preparation was centrifuged for 20 min at 40000*g*, 4 °C. The supernatant was brought on a Sepharose 6B gel filtration column in buffer containing 0.2 M glycine, 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.8, 4 °C), and 10^{-5} M dithiothreitol. The column was eluted at a flow rate of 5 mL h^{-1} cm^{-2}. Peak fractions of the first two peaks were pooled and concentrated by ultrafiltration. Prolyl 4-hydroxylase was obtained in homogeneous form as judged from polyacrylamide gel electrophoresis. Yield in this procedure was 14 mg of protein.

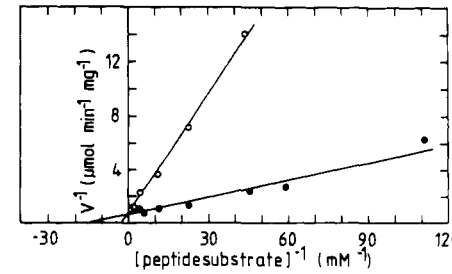

FIGURE 2: Double-reciprocal plot of substrate concentration and initial hydroxylation velocity for (Pro-Pro-Gly)$_5$ and the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$. Prolyl 4-hydroxylase activity was measured polarographically with (Pro-Pro-Gly)$_5$ (open symbols) and the photoaffinity label *N*-(4-azido-2-nitrophenyl)-glycyl-(Pro-Pro-Gly)$_5$ (closed symbols) as substrates. The lines through the experimental points were calculated by means of linear-regression analysis.

RESULTS

Properties of NAG-(Pro-Pro-Gly)$_5$. We found that the photoaffinity label NAG-(Pro-Pro-Gly)$_5$ is a good substrate for prolyl 4-hydroxylase. Figure 2 shows that NAG-(Pro-Pro-Gly)$_5$ and its parent substrate (Pro-Pro-Gly)$_5$ are hydroxylated with the same V, whereas the affinity is higher for NAG-(Pro-Pro-Gly)$_5$ than for (Pro-Pro-Gly)$_5$, K_m values being 77 and 355 μM, respectively.

Photoinhibition by NAG-(Pro-Pro-Gly)$_5$. Prolyl 4-hydroxylase activity is inhibited by illumination in the presence of NAG-(Pro-Pro-Gly)$_5$. Figure 3 shows the enzymic activity remaining after illumination, related to the activity of non-illuminated incubations. Light-induced inactivation is shown

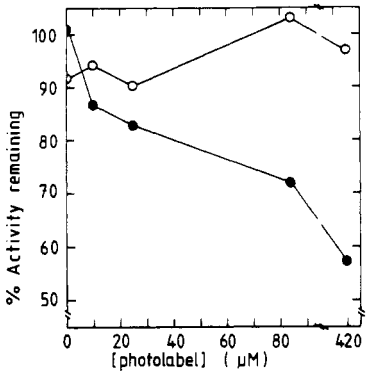

FIGURE 3: Light-induced inactivation of prolyl 4-hydroxylase activity with the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$. Prolyl 4-hydroxylase activity, measured as O_2 consumption, was determined before and after illumination of enzyme in the presence of various concentrations of photoaffinity label in the absence (closed symbols) or the presence (open symbols) of 20 mM (Pro-Pro-Gly)$_5$. Activity is given a as percentage related to nonilluminated samples.

to be prevented by the addition of 20 mM (Pro-Pro-Gly)$_5$. Maximal inhibition that could be reached in one illumination was 40%, necessitating removal of photolyzed NAG-(Pro-Pro-Gly)$_5$ and subsequent illumination in the presence of fresh NAG-(Pro-Pro-Gly)$_5$ in order to obtain a higher degree of inactivation.

Enzymic activity is not affected by incubation with NAG-(Pro-Pro-Gly)$_5$ without illumination, incubation with previously photolyzed NAG-(Pro-Pro-Gly)$_5$, or illumination in the absence of NAG-(Pro-Pro-Gly)$_5$ (results not shown).

The data in Figure 3 were obtained from incubations in the presence of all the constituents of the enzymic activity determination (see Experimental Procedures) except the omission of (Pro-Pro-Gly)$_5$ and the substitution of 20 μM 2,4-pyridinedicarboxylate, which is not decarboxylated by the enzyme (Majaama et al., 1984), for 2-oxoglutarate. As there were no detectable differences in photoinhibition when 2,4-pyridinedicarboxylate, or all substrates and cofactors, was omitted (results not shown), all further incubations were done in buffer as described under Experimental Procedures.

Distribution of Photoaffinity Label among the Subunits. Figure 4 shows the distribution of covalently bound [2-^{3}H]-NAG-(Pro-Pro-Gly)$_5$ among the subunits of prolyl 4-hydroxylase. Only the 64-kDa α subunit is labeled. The radioactivity appears to have a lower mobility on the gel than the protein. This is because the molecular weight of the labeled subunits is actually increased by the 1.6 kDa of the photoaffinity label.

Relation between Labeling and Inhibition. Enzyme preparations were inactivated to different extents by varying the number of illuminations in the presence of [2-^{3}H]NAG-(Pro-Pro-Gly)$_5$. The enzyme was separated into subunits by polyacrylamide gel electrophoresis, and the gels were sliced and counted for ^{3}H. The amount of photoaffinity label covalently linked to the α subunit in relation to the enzymic activity is shown in Figure 5. There is a linear relationship between inhibition of enzymic activity and amount of bound photoaffinity label. Extrapolation of the data to 100% inhibition yields a value of close to 2 mol of bound photoaffinity label per mole of prolyl 4-hydroxylase, corresponding to one binding site per α subunit.

Reaction Kinetics of Photoinhibited Enzyme. In order to study the kinetics of the inhibited prolyl 4-hydroxylase, we prepared enzyme that was illuminated for 5 cycles in the presence of 210 μM NAG-(Pro-Pro-Gly)$_5$. This resulted in 90% inactivation. As a control, enzyme was illuminated in

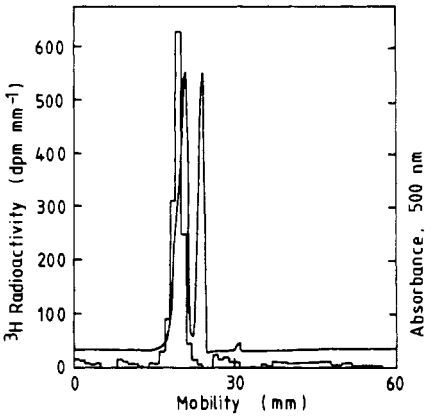

FIGURE 4: Distribution pattern of radioactivity after NaDodSO$_4$ gel electrophoresis of prolyl 4-hydroxylase photolabeled with *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$ to the extent of 0.33 mol of label/mol of enzyme. Prolyl 4-hydroxylase was separated into subunits by gel electrophoresis in the presence of NaDodSO$_4$. The gel was stained with Coomassie Brillian Blue and destained. The position of protein in the gel was measured by scanning the gel at 500 nm (continuous line). Radioactivity was determined by extraction of gel slices with Protosol and counting in a liquid scintillation counter (histogram).

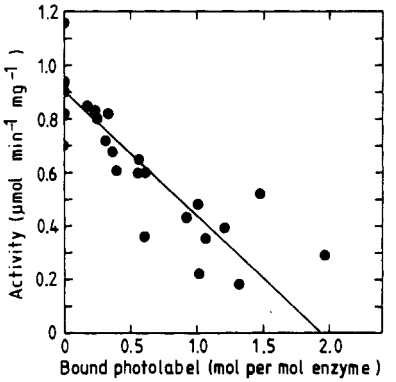

FIGURE 5: Relation between amount of covalently bound photoaffinity label and enzymic activity of prolyl 4-hydroxylase after light-induced inactivation with the photoaffinity label *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$. Prolyl 4-hydroxylase was inactivated to various degrees by repeated cycles of illumination in the presence of photoaffinity label, to a maximum of five cycles. Radioactivity was measured in α subunits, separated from other protein and noncovalently bound photoaffinity label by gel electrophoresis. Enzymic activity was measured as the release of $^{14}CO_2$ from 2-oxo-[1-^{14}C]glutarate. The line through the experimental points ($y = 0.903 - 0.467x$) was calculated by means of geometric mean regression analysis as described by Sokal & Rohlf (1981). The 95% confidence limits for the slope are −0.364 and −0.543. Correlation coefficient $r = -0.854$.

the presence of 210 μM (Pro-Pro-Gly)$_5$. These preparations were subjected to gel filtration on a Sepharose 6B column in order to remove noncovalently bound NAG-(Pro-Pro-Gly)$_5$ (see Experimental Procedures).

To investigate whether and to what extent photoinhibited enzyme is still able to decarboxylate 2-oxoglutarate, either uncoupled, coupled to the hydroxylation of covalently bound NAG-(Pro-Pro-Gly)$_5$, or both, we measured also the enzymic activity in the absence of added (Pro-Pro-Gly)$_5$. Under these conditions, the activity of the enzyme preparation illuminated with NAG-(Pro-Pro-Gly)$_5$ was 4.2 nmol min^{-1} (mg of protein)$^{-1}$, compared with 4.8 nmol min^{-1} (mg of protein)$^{-1}$ for the control. The former value may be an overestimation, for there may have been decarboxylation by free enzyme of 2-oxoglutarate coupled to the hydroxylation of covalently enzyme-bound nitreno derivative of NAG-(Pro-Pro-Gly)$_5$. However, with 2 mol of photoaffinity label/mol of enzyme,

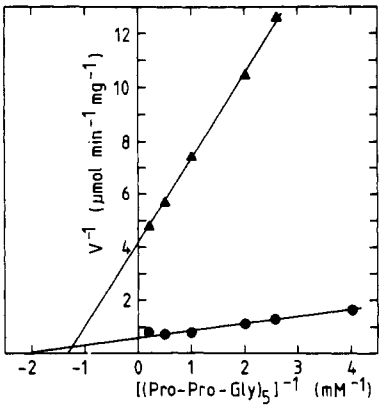

FIGURE 6: Double-reciprocal plot of substrate concentration and initial hydroxylation velocity of prolyl 4-hydroxylase inactivated by illumination in the presence of the photoaffinity label *N*-(4-azido-2-nitrophenyl)glycyl-(Pro-Pro-Gly)$_5$. Prolyl 4-hydroxylase was subjected to five cycles of light-induced inactivation with the photoaffinity label (triangles). As a control, prolyl 4-hydroxylase was illuminated for five cycles in the presence of (Pro-Pro-Gly)$_5$ (circles). Enzymic activity was measured as the release of $^{14}CO_2$ from 2-oxo[1-^{14}C]glutarate. The lines through the experimental points were calculated by means of linear-regression analysis.

the concentration of peptide in the incubation would not exceed 3.3×10^{-7} M, which is very low compared to the K_m for (Pro-Pro-Gly)$_5$ (3.5×10^{-4} M). Therefore, it is not likely that more than 10% of the total amount of decarboxylated 2-oxoglutarate has been coupled to the hydroxylation of covalently enzyme-bound nitreno derivative of NAG-(Pro-Pro-Gly)$_5$ by free enzyme. We conclude that decarboxylation of 2-oxoglutarate in the absence of added (Pro-Pro-Gly)$_5$, in the contrast to the coupled reaction in the presence of this peptide substrate, is not strongly inhibited by photolabeling with NAG-(Pro-Pro-Gly)$_5$. This indicates that the bound photolabel does not interfere with the binding of the substrates (O_2, 2-oxoglutarate, and ascorbate) and cofactor (Fe^{2+}) involved in the uncoupled reaction. The integrity of the structure in the enzyme that is responsible for oxidative decarboxylation of 2-oxoglutarate, with the concomitant creation of a transient oxygen-transferring intermediate, is apparently not affected by the bound photolabel. Consequently, the inhibition of peptide substrate hydroxylation must be because the bound photolabel prevents the peptide substrate from binding at the catalytic site. Figure 6 is a Lineweaver–Burk plot of the activities measured with variable (Pro-Pro-Gly)$_5$ concentration. It is evident from this graph that inhibited enzymic activity cannot be restored by high (Pro-Pro-Gly)$_5$ concentration. V is decreased by 85%, whereas the K_m is scarcely affected.

Binding on Other Sites Than the Catalytic Center. The photoinactivated enzyme retained up to 8 mol of photolyzed noncovalently bound NAG-(Pro-Pro-Gly)$_5$ per mole of enzyme after centrifugation through Sephadex. The noncovalently bound label could be removed by equilibrium gel filtration on Sepharose 6B (see Experimental Procedures). Under the same conditions without enzyme, all the photoaffinity label was retained in the Sephadex. This indicates that peptide binding sites additional to the two catalytic sites are present and that only the latter are blocked by the covalently bound photoprobe. To investigate further the peptide-binding capacity of an enzyme fraction with both its catalytic sites blocked by photoaffinity label, we made a 64–% inhibited preparation of prolyl 4-hydroxylase bearing overall 1.1 mol/mol covalently bound and 8 mol/mol noncovalently bound photoaffinity label. We assume that the chance for an α subunit to be photolabeled is independent of whether or not the other α subunit in the enzyme molecule is photolabeled. This assumption is justified

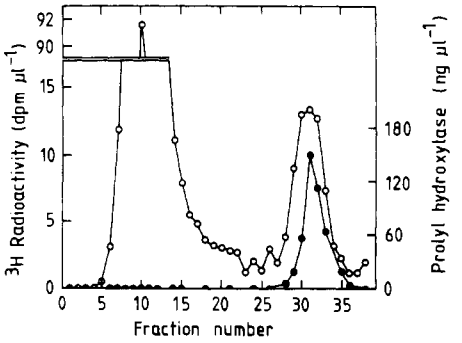

FIGURE 7: Binding of prolyl 4-hydroxylase with covalently bound photoaffinity label on a poly(L-proline) affinity column. Prolyl 4-hydroxylase was subjected to three cycles of light-induced inactivation with the photoaffinity label *N*-(4-azido-2-nitrophenyl)[2-^{3}H]-glycyl-(Pro-Pro-Gly)$_5$ and applied to a poly(L-proline) affinity column. The column was eluted with buffer (fractions 1–15) and with a gradient of low molecular weight poly(L-proline) (from fraction 15). The enzyme preparation contained 8 mol of noncovalently bound photoaffinity label and 1.1 mol of covalently bound photoaffinity label per mole of enzyme and was inhibited by 64%. The radioactivity (open symbols) in the fractions was measured by liquid scintillation counting. The amount of prolyl 4-hydroxylase (closed symbols) was measured by rocket electrophoresis (Weeke, 1973). The validity of this method for photolabeled enzyme was tested with a 90% inactivated enzyme preparation. The enzyme was immunoreactive but yielded slightly diminished peak areas. The gel contained 1 wt % agarose, 0.1 wt % Triton X-100, and 0.25 vol % antiserum, in electrophoresis buffer containing 100 mM glycine and 38 mM Tris-HCl (pH 8.6). Electrophoresis was for 4 h at 5.5 V/cm.

by the linearity of the relationship between inhibition and amount of bound photolabel in Figure 5 and the not significantly altered K_m for (Pro-Pro-Gly)$_5$ in inhibited enzyme as shown in Figure 6. A population of enzyme molecules that has 1 mol of bound photoaffinity label per mole of tetrameric enzyme can thus be considered to consist of 25% of the molecules with two molecules photoaffinity labeled, 50% with one molecule labeled, and 25% nonlabeled. We applied the 64–% inhibited enzyme at 4 °C to an affinity column of poly(L-proline) of M_r 40 000 (a competitive inhibitor toward the peptide substrate) coupled to Sepharose 4B. After the column was washed with buffer (containing 0.2 M glycine, 0.2 M NaCl, 10^{-5} M dithiothreitol, and 0.05 M Tris-HCl, pH 7.8 at 4 °C), a gradient of poly(L-proline) (M_r 8000) from 0 to 5 mg/mL was applied to elute the bound enzyme. Radioactivity as well as prolyl 4-hydroxylase content was measured in the fractions. The results are presented in Figure 7. The first peak of radioactivity is not accompanied by prolyl 4-hydroxylase. This represents noncovalently bound photolyzed affinity label that is released by the enzyme upon binding to the poly(L-proline) of the affinity column. At fraction 15, a gradient of poly(L-proline) was started. This releases a second peak of radioactivity, coinciding with a peak of prolyl 4-hydroxylase and thus representing photolabeled enzyme. The second peak of radioactivity contained 98% of the radioactivity covalently bound to the enzyme, as judged from the radioactivity remaining after polyacrylamide gel electrophoresis. This quantitative recovery, combined with the absence of prolyl 4-hydroxylase in the first peak, is evidence for poly(L-proline) binding capacity in enzyme molecules in which the two catalytic binding sites are occupied by photoaffinity label. Therefore, other binding sites than those at the catalytic site must exist.

Discussion

There are several possibilities to account for the higher affinity of prolyl 4-hydroxylase for longer peptide substrates.

Among these are cooperativity in binding of prolyl 4-hydroxylase to the peptide substrate, processive hydroxylation, and a large binding site for peptides.

There are no kinetic data that indicate cooperative binding of a number of enzyme molecules to the same substrate molecule. That a large peptide can act as a multifunctional reagent can not by itself account for a higher affinity, since this still exists when expressed per weight of substrate instead of in moles (Prockop et al., 1976).

As shown here, the enzyme contains peptide binding subsites that allow the binding to poly(L-proline), a competitive inhibitor toward the peptide substrate, even when both catalytic binding sites are blocked by the covalently bound nitreno derivative of NAG-(Pro-Pro-Gly)$_5$. At this stage, the conception of a catalytic binding site and a binding subsite may require further explanation. Since the tripeptide X-Pro-Gly represents the minimal requirement for peptidyl proline to become hydroxylated, we define the catalytic binding site as a site where such a tripeptide, either as such or as part of a larger peptide, becomes hydroxylated. On the other hand, binding to a subsite will not result in hydroxylation of that particular part of the peptide involved in this binding.

The existence of binding subsites may account for the effect of chain length of a peptide substrate on its K_m. A mechanism is conceivable in which several hydroxylations, not necessarily in consecutive -X-Pro-Gly- triplets, take place during one encounter with the enzyme and a relatively long substrate, such as the enzyme's physiological substrate procollagen. In such a mechanism, the enzyme remains bound to the substrate by its subsites for a number of catalytic cycles.

The question arises why it is impossible to photolabel more than two binding sites per enzyme although up to 8 mol of NAG-(Pro-Pro-Gly)$_5$ can be bound noncovalently per mole of enzyme. The nitreno group formed upon photoactivation reacts with any C–H or O–H bond in its vicinity, and as a consequence, a number of nitreno groups has reacted with water to form a hydroxylamine before they can attack the enzyme (Bayley & Knowles, 1977). It is possible that the binding subsites occupied by the noncovalently bound photolabel are in a water-rich environment on the enzyme surface. On the other hand, the two catalytic sites, and possibly binding subsites in their near vicinity, may be situated in a cleft on the enzyme, allowing these sites to be photolabeled more efficiently, with concomitant loss in enzyme activity.

In addition to the active form of prolyl 4-hydroxylase, which is a tetramer of 240 kDa with $\alpha_2\beta_2$ subunit structure, a protein without enzymic activity, resembling the β subunit, can be found in cells (McGee et al., 1971; Chen-Kiang et al., 1977). This protein, which cross reacts with antibodies raised against the tetrameric enzyme, is termed cross-reacting protein or β' in the literature. It has been suggested that it acts as a precursor for the active enzyme (Berg et al., 1980; Majaama et al., 1979; Majaama & Oikarinen, 1982), but this does not explain why the cross-reacting protein is sometimes present in cells in great excess over the tetrameric enzyme and some molecules are turned over without ever being assembled in an active enzyme (Majaama et al., 1979). It may be that the cross-reacting protein has an additional function. In this context, it is of interest to know the function of the β subunit in the active enzyme. We found that the photoaffinity label is exclusively associated with the α subunit of prolyl 4-hydroxylase. We are not certain whether this means that the catalytic center is located on the α subunit. (Pro-Pro-Gly)$_5$ is preferentially hydroxylated in the penultimate triplet from its carboxy terminus. Since the photoreactive group of NAG-(Pro-Pro-Gly)$_5$ is on the amino terminus, it may react on a site far away from the catalytic center. Recently, Höythyä et al. (1984) reported a monoclonal antibody against the β subunit of human prolyl 4-hydroxylase that was capable of inhibiting enzymic activity and cross reacted with enzyme from other species, suggesting that the catalytic center is located on the β subunit. Our aim is to further characterize the vicinity of the catalytic center by means of photoaffinity labels of different chain length and with the position of the photoreactive group at the carboxy terminus of the peptide as well as at the amino terminus. In a preliminary experiment, we found for the photoaffinity label *N*-(4-azido-2-nitrophenyl)[2-^{3}H]glycyl-(Pro-Pro-Gly)$_5$ that it appeared in three discrete peptides after CNBr peptide digestion (not shown).

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Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Fluorescent Affinity Labeling of the Catalytic Subunit from Bovine Skeletal Muscle with *o*-Phthalaldehyde†

Rajinder N. Puri,* Deepak Bhatnagar, and Robert Roskoski, Jr.
Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70119
Received March 27, 1985

ABSTRACT: The catalytic subunit of adenosine cyclic 3′,5′-monophosphate dependent protein kinase from bovine skeletal muscle was rapidly inactivated by *o*-phthalaldehyde at 25 °C (pH 7.3). The reaction followed pseudo-first-order kinetics, and the second-order rate constant was 1.1×10^2 M^{-1} s^{-1}. Absorbance and fluorescence spectroscopic data were consistent with the formation of an isoindole derivative (1 mol/mol of enzyme). The reaction between the catalytic subunit and *o*-phthalaldehyde was not reversed by the addition of reagents containing free primary amino and sulfhydryl functions following inactivation. The reaction, however, could be arrested at any stage during its progress by the addition of an excess of cysteine or less efficiently by homocysteine or glutathione. The catalytic subunit was protected from inactivation by the presence of the substrates magnesium adenosine triphosphate and an acceptor serine peptide substrate. The decrease in fluorescence emission intensity of incubation mixtures containing iodoacetamide- or 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine-modified catalytic subunit and *o*-phthalaldehyde paralleled the loss of phosphotransferase activity. Catalytic subunit denatured with urea failed to react with *o*-phthalaldehyde. Inactivation of the catalytic subunit by *o*-phthalaldehyde is probably due to the concomitant modification of lysine-72 and cysteine-199. The proximal distance between the ε-amino function of the lysine and the sulfhydryl group of the cysteine residues involved in isoindole formation in the native enzyme is estimated to be approximately 3 Å. The molar transition energy of the catalytic subunit–*o*-phthalaldehyde adduct was 121 kJ/mol and compares favorably with a value of 127 kJ/mol for the 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole in hexane, indicating that the active site lysine and cysteine residues involved in formation of the isoindole derivative of the catalytic subunit are located in a hydrophobic environment. *o*-Phthalaldehyde probably acts as an active site specific reagent for the catalytic subunit.

A large number of physiological effects of adenosine cyclic 3′,5′-monophosphate (cAMP)[1] in eukaryotic organisms are the result of phosphorylation of specific cellular proteins by cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (Krebs, 1972; Rubin & Rosen, 1975; Rosen et al., 1977; Cohen, 1978; Krebs & Beavo, 1979), which are widely distributed in nature (Kuo & Greengard, 1969). The inactive holoenzyme consists of two identical regulatory subunits (R) and two catalytic subunits (C) held together by noncovalent interactions (Krebs & Beavo, 1979). The tetrameric inactive enzyme undergoes dissociation in the presence

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; C subunit, catalytic subunit of type II cAMP-dependent protein kinase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; EA, 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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