Acta Chemica Scandinavica B 31 (1977) 604—608

Pseudomonas Cytochrome *c* Peroxidase. XIII. pH-Denaturation of the Enzyme

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The effect of pH on the oxidized *Pseudomonas* cytochrome *c* peroxidase molecule was studied by measuring the peroxidatic activity, the sedimentation velocity, the circular dichroic spectra in the far UV and Soret regions, and the optical absorption spectra of the enzyme in the pH range 2.5 – 13.0 at a constant ionic strength ($\mu = 0.1$). The enzyme was stable in a narrow pH region, pH 6.0 – 7.4. In the low pH range the gross tertiary structure was observed to change quite simultaneously with the enzymatic activity and secondary structure. The optical absorption spectra indicated that there were no coordinated internal protein ligands in the 6th coordination positions of the heme prosthetic groups at the lowest pH studied. In the high pH range the secondary structure and the protein environment of hemes were observed to remain stable after the tertiary structure had changed and the activity had decreased. According to the optical absorption spectra the 6th internal protein ligands of hemes were retained at the highest pH studied.

Pseudomonas cytochrome *c* peroxidase (cytochrome *c*:H_2O_2-oxidoreductase, EC 1.11.1.5, PaCCP) is a bacterial peroxidase containing two molecules of covalently bound heme *c* as prosthetic groups in a single polypeptide chain of 354 amino acids.[1–4] The molecule contains no disulfide bonds,[3] it has an excess of hydrophobic amino acids,[4] and its isoelectric point is 6.7.[1] The optical absorption spectra of PaCCP show the low spin or hemochrome structure of the prosthetic groups, that is with internal protein ligands at the 5th and 6th coordination positions of the heme iron,[1,5] as in *c*-type cytochromes. The frictional ratio calculated from the hydrodynamic properties of the molecule indicates that PaCCP is quite a symmetric molecule, differing little from a spherical one.[2,6]

PaCCP catalyzes specifically the hydrogen peroxide oxidation of two macromolecular substrates, *Pseudomonas* respiratory chain proteins cytochrome *c*-551 and copper protein azurin.[7]

In the present work the effect of pH on the oxidized Fe(III) – PaCCP molecule was studied in the pH range 2.5 – 13.0 at a constant ionic strength ($\mu = 0.1$).

The pH-stability of the enzyme, determined by measuring the peroxidatic activity, and the effect of high pH on the optical absorption spectra of the enzyme have been studied earlier.[1,5]

MATERIALS AND METHODS

Pseudomonas cytochrome c peroxidase was prepared from the acetone-dried cells of *P. aeruginosa* as previously described.[8] The preparation was homogeneous in disc electrophoresis (pH 8.6, 7 % gel,[9] staining with Coomassie Brilliant Blue G-250 [10]) and in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (10 % gel).[11] After the purification procedure the enzyme was in the oxidized Fe(III) form. The absorbance ratio A_{407}/A_{280} of the preparation was 4.6. The concentration of the enzyme was determined spectrophotometrically using E(1 %, 1 cm) equal to 12.1 at 280 nm.[2] The molar concentration of the enzyme was calculated using a molecular weight of 43 200 based on the iron content of the enzyme.[2,3]

Denaturation of the enzyme. A concentrated stock solution of PaCCP was diluted with buffer and the diluted solution was dialyzed against the same buffer for 3 h at room tem-

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perature. The dialyzed enzyme solution was used in all the measurements described. The pH of the solution was checked after the measurements. The buffers ($\mu = 0.1$) used were: glycine–HCl buffer pH 2.5, sodium acetate buffers pH 3.8–5.3, sodium phosphate buffers pH 6.0–8.0, TRIS-HCl buffer pH 9.0, sodium borate buffers pH 9.3–9.8, and 0.1 M sodium hydroxide pH 13.0.

Peroxidatic activity was assayed spectrophotometrically by measuring the rate of the peroxidatic oxidation of reduced *Pseudomonas* cytochrome *c*-551 as previously described.[5] The reaction was initiated by mixing 2–5 µl of suitably diluted enzyme with 2.0 ml of the reaction mixture. The enzyme was diluted with the buffer used in the denaturation. The activity was expressed in terms of arbitrary units (decrease in absorbance per 10 s per 10 µl of enzyme solution) calculated from the slope of the reaction curve.

Ultracentrifugation analyses. The sedimentation velocities were measured in a Beckman-Spinco Model E analytical ultracentrifuge at a rotor speed of 59 870 rpm at 20 °C as previously described.[2]

Spectropolarimetry. Circular dichroic (CD) spectra were recorded on a Cary 61 spectropolarimeter at 25 °C. Cells with fused quartz windows and path lengths 0.1–1 mm were used. Path lengths were dictated by the absorbance of the solution. The stability of each preparation during the CD measurements was confirmed by recording the optical absorption spectra immediately before and after each CD measurement. Base lines were recorded and subtracted from the dichroic curves. The ellipticities above 250 nm are expressed as molar ellipticities, $[\theta]$, in degrees cm²/dmol. Ellipticities below 250 nm are expressed as mean residual ellipticities, $[\theta]_{MRW} = [\theta]/n$, where n, the number of residues per molecule, is 354 for PaCCP.

Spectrophotometry. Spectrophotometric concentration determinations were performed with a Beckman DU-2 spectrophotometer, peroxidatic activities were measured with a Beckman DK-1 A recording spectrophotometer and optical absorption spectra with a Cary 15 recording spectrophotometer, all at 25 °C.

pH-Measurements were carried out at 25 °C with a Radiometer PHM 3 pH-meter standardized against Beckman buffer pH 7 No. 3581, 0.05 M potassium phthalate buffer pH 4.01 and 0.01 M $Na_2B_4O_7.10H_2O$ pH 9.18.

Chemicals. Sodium dodecyl sulfate (pract., Fluka) and urea (pure, Merck) were recrystallized before use. Other reagents were of analytical grade.

RESULTS

The peroxidatic activity of the enzyme was measured in the pH range 2.5–13.0, and shown in Fig. 1, expressed as the percentage activity of the native PaCCP left after the incubation at different pH values.

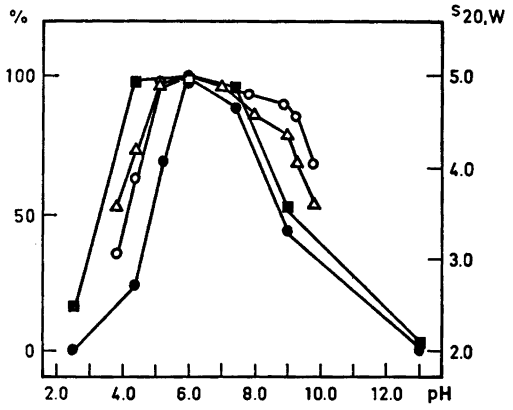

Fig. 1. The pH dependence of (●) the peroxidatic activity of *Pseudomonas* cytochrome *c* peroxidase (PaCCP) as percent of that of the native enzyme; (■) the sedimentation coefficient $s_{20,w}$ of PaCCP; (△) the change of the mean residual ellipticity of PaCCP at 208 nm expressed as percent of the total change caused by 8 M urea at pH 6.0; and (○) the change of the molar ellipticity of PaCCP at 412 nm expressed as percent of the total change caused by 8 M urea at pH 6.0.

The sedimentation coefficient, $s_{20,w}$, of PaCCP was determined in the pH range 2.5–13.0. The value of the sedimentation coefficient decreased from that of the native enzyme, 5.0 S, to 2.5 S at the lowest pH and to 2.0 S at the highest examined (Fig. 1). The protein sedimented as a single boundary at every pH.

Circular dichroic spectra of PaCCP in the far UV (200–250 nm) and the near UV (Soret, 370–450 nm) regions in the pH range 3.8–9.8 as well as in 8 M urea at pH 6.0 are shown in Fig. 2.

The native PaCCP (pH 6.0) has a prominent negative ellipticity band at 208 nm with a shoulder at 223 nm. Characteristic of the spectra in the far UV region in both the low and the high pH regions is the decrease of the negative ellipticity at 208 nm. In 8 M urea the shape of the band has totally changed and the band at 223 nm has disappeared. The change of the mean residual ellipticity at 208 nm expressed as a percentage of the total change caused by 8 M urea (pH 6.0), which describes

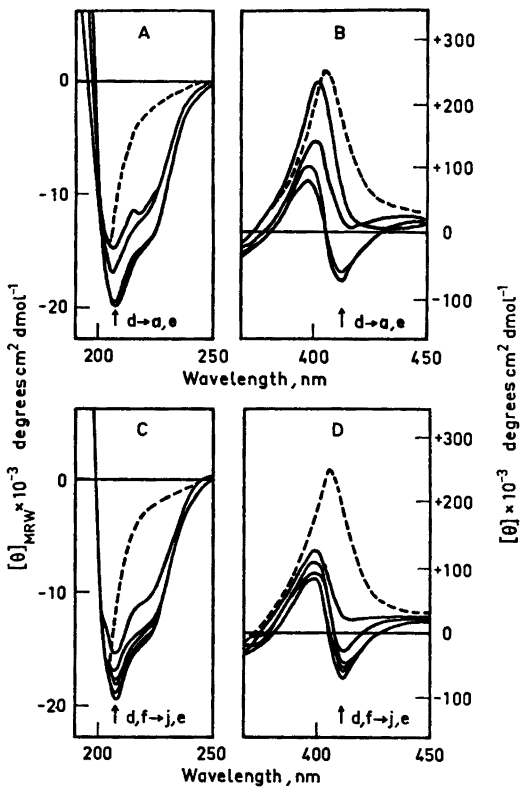

Fig. 2. Circular dichroic spectra of *Pseudomonas* cytochrome *c* peroxidase (47 μM) in the pH region 3.8 – 9.8 in the far ultra-violet (*A*, *C*) and the Soret (*B*, *D*) region. The curves can be distinguished at 208 and 412 nm. (*a*) pH 3.8, (*b*) pH 4.4, (*c*) pH 5.1, (*d*) pH 6.0, (*e*, the broken line) pH 6.0, 8 M urea, (*f*) pH 7.0, (*g*) pH 8.0, (*h*) 9.0, (*i*) pH 9.3, and (*j*) pH 9.8.

the change of the CD spectrum in the far UV region, is shown for the pH range 3.8 – 9.8 in Fig. 1.

At the Soret region the native PaCCP (pH 6.0) has a positive ellipticity band at 400 nm and a negative one at 412 nm. The negative band totally disappears at both low and high pH and the spectrum approaches that of PaCCP in 8 M urea, with one positive band at 407 nm. The change in the molar ellipticity at 412 nm expressed as a percentage of the total change caused by 8 M urea (pH 6.0) which describes the change of the CD spectrum in the Soret region, is shown for the pH range 3.8 – 9.8 in Fig. 1. The change of the CD spectrum in this region is more prominent at low than at high pH. The spectrum of the urea-treated PaCCP was achieved neither by acid (pH 3.8) nor alkaline (pH 9.8) denaturation.

Optical absorption spectra of PaCCP were recorded in the pH range 2.5 – 13.0. Of the visible spectra (350 – 650 nm) measured, the spectrum of the native enzyme at pH 6.0 and the spectra of the denatured enzyme at pH 2.5 and pH 13.0 are shown in Fig. 3.

The characteristic features in the spectrum at low pH are the Soret band at 397 nm with a reduced absorbance compared with that of the native enzyme, reduced absorbance in the 450 – 650 nm region, and new slight maxima around 510 and 625 nm. At high pH a δ-band at 353 nm is observed, the Soret band with increased absorbance has changed to 410 nm, the absorbance at 450 – 650 nm has slightly increased and a shoulder at around 568 nm has appeared.

After denaturation experiments every preparation was checked by *polyacrylamide gel electrophoresis* in the presence of sodium dodecyl sulfate. Only the same polypeptide band as in the untreated PaCCP, with an apparent molec-

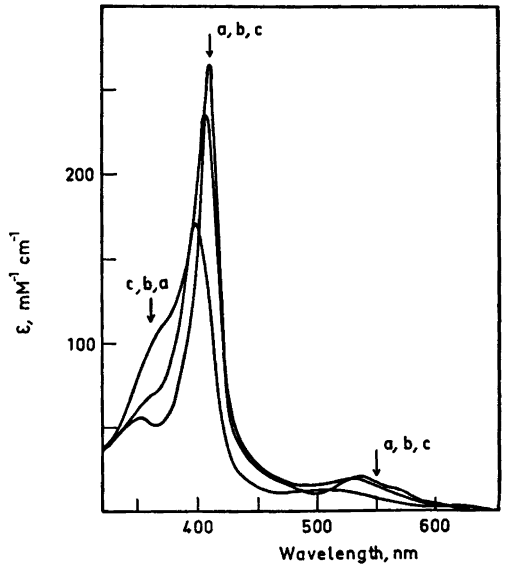

Fig. 3. Optical absorption spectra of oxidized *Pseudomonas* cytochrome *c* peroxidase (47 μM) at (*a*) pH 13.0, (*b*) pH 6.0, and (*c*) pH 2.5 in the visible region. The curves can be distinguished at 360, 407 and 550 nm.

ular weight of 40 000,[3] was observed in each of the preparations.

DISCUSSION

The peroxidatic activity of PaCCP was the most sensitive of the properties measured to changes of pH (Fig. 1). Earlier the enzyme was shown to be stable at pH 6.0,[1] and at this pH it is assumed to be in the native form. The activity decreased rapidly in the acid region and was somewhat more stable in the alkaline region but decreased markedly when the pH was over 7.4. Accordingly to the present study the pH region where the enzymatic activity is stable is quite narrow, pH 6.0 – 7.4. A slightly different pH region, pH 5 – 7, for the stability was observed earlier.[7] Then, however, the activity was measured at pH 7.2 and not at the optimum pH 6.0 as in the present study; this may explain the difference. Peroxidatic activity is lost after only minor changes in the molecule, and before any changes could be observed by the physical methods used in this study. A macromolecular substrate such as *Pseudomonas* cytochrome *c*-551 may demand quite a large area of the enzyme molecule to be unchanged, that is in the native form, for the formation of enzyme-substrate complex.

The sedimentation coefficient obtained from the ultracentrifuge analysis is a measure of the frictional coefficient of the molecule and it depends on the radius of the hydrodynamic particle. The sedimentation coefficient thus describes the folding of the polypeptide chain or the tertiary structure of the protein molecule. It offers a means of following the transition of the molecule from the native to the denaturated state.[12] The sedimentation coefficient of PaCCP remained unchanged to a lower pH than any other property measured, indicating the retention of the gross tertiary structure in the low pH region. Some changes in the molecule have occurred, as seen from the activity and circular dichroic data (Fig. 1). In the high pH region the sedimentation coefficient changed simultaneously with the loss of the enzymatic activity (Fig. 1). PaCCP sedimented as a single boundary at every pH studied. This confirms an earlier report that the molecule consists of one polypeptide chain [3] and has no subunits. The value of the sedimentation coefficient approached 2 S at low as well as at high pH, indicating the increase in the effective hydrodynamic volume caused by the unfolding of the polypeptide chain. The covalent structure of the polypeptide backbone was preserved, as shown by the polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in which the single protein band had the same apparent molecular weight as the native enzyme.

The circular dichroic spectrum of a protein in the far UV region (180 – 250 nm) offers a means of investigating the conformation of the polypeptide backbone or the secondary structure of the protein molecule, assuming that the contributions of non-peptide chromophores in this region are insignificant.[12,13] In the low pH region the secondary structure of PaCCP, according to the far UV CD spectrum, changed quite simultaneously with the tertiary structure measured by the sedimentation coefficient. It prevailed to some extent after the enzymatic activity had decreased (Fig. 1). In the high pH region the far UV CD spectra show that the secondary structure changed later than the tertiary structure. Theoretical CD curves have been published for the three conformations, helix, β-pleated sheet and unordered, based on the data from five reference proteins.[13] When the spectra of PaCCP are compared qualitatively with the theoretical spectra it seems that PaCCP contains helical as well as β-structure and/or unordered structure. The helical structure decreased at low as well as high pH, as seen from the change of ellipticity at 223 nm (Fig. 2). The CD spectra of PaCCP at low and high pH were also compared with those in 8 M urea at pH 6.0. In 8 M urea PaCCP was mainly in the random coil unordered form, as seen from the spectrum (Fig. 2). The pH-denaturation at either low or high pH did not result in the loss of ordered structure to the same extent as in 8 M urea.

The dichroic band at 370 – 450 nm (the Soret region) is influenced by the effect of the protein environment on the heme transitions, although the heme chromophore itself has no CD band in the Soret region.[14,15] In the low pH region the CD bands of PaCCP in the Soret region changed in a similar way to the far UV CD-band (Fig. 1), that is the heme environment changed simultaneously with the changes

in the secondary structure of the molecule. In the high pH region the heme environment remained unchanged further than the enzymatic activity and the tertiary structure measured by $s_{20,w}$ (Fig. 1). The disappearance of the complex nature of the CD band in the Soret region at low and high pH, as well as in 8 M urea (Fig. 2) indicates that both the two heme groups are in different asymmetric positions in the native molecule and change to identically asymmetric positions in the denaturated molecule. A similar simplification of CD spectra of horse heart cytochrome *c* by urea and other denaturants has been observed and attributed to the opening of the heme crevice and changes in heme ligands.[16,17]

The optical absorption spectra of hemoproteins in the near UV (Soret) and visible wavelength regions depend on the electronic and magnetic structure of heme and reflect the state of the prosthetic group. The optical absorption spectra of Fe(III)-PaCCP at the lowest and highest pH examined are totally different (Fig. 3). At low pH the spectrum shows the characteristics of a high-spin, myoglobin type spectrum,[18] one observed for example in the so-called high-spin cytochromes[19,20] and in horse heart cytochrome *c* below pH 2.5.[21] The "high-spin spectrum" indicates that the internal strong protein ligand is no longer coordinated in the 6th coordination position of the heme iron. At high pH the low-spin type spectrum of Fe(III)-PaCCP is preserved (Fig. 3). The stability of the low-spin structure of the reduced, Fe(II) – PaCCP in the alkaline region has been shown earlier.[5] Thus, in contrast to the acid denaturation, even severe alkaline denaturation does not lead to the loss of the 6th internal strong protein ligands of heme irons. The ligands may have a stabilizing effect on the heme environment in the high pH region observed in CD spectra.

According to the above results Fe(III)-PaCCP is denatured easily at both low and high pH. It may be therefore classified, according to Tanford,[12] with those proteins that are drastically altered when pH is changed.

Acknowledgement. We wish to thank Professor N. Ellfolk for his advice and interest during the investigation.

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Received March 18, 1977.