ACTA CHEMICA SCANDINAVICA 27 (1973) 2193–2198

Pseudomonas Cytochrome *c* Peroxidase

VIII. The Amino Acid Composition of the Enzyme

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The amino acid composition of *Pseudomonas* cytochrome *c* peroxidase has been determined. The analyses indicate the presence of the following amino acid residues: Asp_{36}, Thr_{16}, Ser_{22}, Glu_{39}, Pro_{28}, Gly_{29}, Ala_{37}, Val_{24}, Met_4, $Ileu_9$, Leu_{31}, Tyr_8, Phe_{19}, Lys_{23}, His_9, Arg_{13}, Cys_3, Trp_4,$(\text{-CONH}_2)_{35}$. No neutral sugars, amino sugars or sialic acids were detected in the enzyme. The molecular weight of 44 047 computed from the amino acid composition corresponds to the values based on the iron and heme content. A value of 0.711 ml/g for the partial specific volume was calculated from the weight percentages of the amino acid residues and their respective specific volumes. The electrophoretic mobility of the enzyme can be satisfactorily explained by the amino acid composition.

Homogeneous cytochrome *c* peroxidase (cytochrome *c*:H_2O_2-oxidoreductase, EC 1.11.1.5) has recently been purified from *Pseudomonas aeruginosa*.[1,2] It differs from plant and animal peroxidases in containing heme *c* (two molecules per mol) as the prosthetic group.[1,3] In the present publication we report the results of the analyses of its amino acid composition and the possible presence of carbohydrates, common constituents of plant and animal peroxidases.

MATERIALS AND METHODS

Pseudomonas cytochrome *c* peroxidase (PsCCP) was prepared from the acetone-dried cells of *P. aeruginosa* as previously described.[1,2] The preparation was homogeneous when analyzed by disc electrophoresis [4] (7 % gel, pH 8.9, staining according to Weber and Osborne [5]) and its absorbance ratio A_{407}/A_{280} was 4.53.

Amino acid analyses. Samples of PsCCP were extensively dialyzed against twice-distilled water before analysis. The acid hydrolysis of the protein was performed in 6 N HCl at 110°C in evacuated sealed Pyrex tubes for periods of 20 and 70 h. To prevent the loss of tyrosine during hydrolysis, 10 μl of 0.1 M phenol was added to the samples.[6] After hydrolysis, HCl was removed in a rotary evaporator. Aliquots containing 0.4 – 0.6 mg hydrolysate were analyzed according to the procedure of Moore *et al.*[7] using a Beckman Spinco Model 120 B amino acid analyzer. A Beckman standard amino acid mixture was used to standardize the columns. The analysis of tryptophan was carried out on samples

of unhydrolyzed protein according to "procedure K" of Spies and Chambers.[8] Cyst(e)ine and methionine were determined in the amino acid analyzer as cysteic acid and methionine sulfone following performic acid oxidation according to Moore.[9] The number of residues was calculated by reference to the molar quantities of amino acids stable to oxidation.

Amide ammonia was determined after the hydrolysis of PsCCP samples in 1 N HCl for 4 h in a stoppered test tube at 100°C. The Conway microdiffusion technique was employed as previously described.[10,11] The ammonia liberated from amide groups and trapped in 0.01 N HCl in the inner chamber of the Conway vessel was determined in the amino acid analyzer.

Total nitrogen content of dried samples of PsCCP was determined using a Coleman 29 A C Nitrogen Analyzer II which is based on the Dumas method.

Carbohydrate analyses. Neutral sugars were analyzed in the unhydrolyzed protein by the Winzler orcinol-sulfuric acid procedure [12] and by the anthrone method.[13] Hexosamine analyses were performed on the neutralized hydrolysate (hydrolysis in 4 N HCl at 100°C for 4 h) by the Rondle and Morgan procedure. [14,15] Sialic acids were analyzed after the hydrolysis of samples in 0.05 M H_2SO_4 at 80°C for 1 h by the thiobarbituric acid method.[16] Optical absorption difference spectra (sample *minus* protein blank) of the reaction products were recorded on a Cary 15 recording spectrophotometer at the appropriate wavelengths. The spectra obtained from PsCCP were compared with those of standard monosaccharides and glycoproteins (lactoperoxidase, prepared from milk according to Carlström,[17] containing 4.1 – 5.4 % neutral sugars and 4.8 % amino sugars,[18] ovalbumin, Grade V, Sigma, containing 2 % neutral sugars and 1.2 % amino sugars,[19] and casein, Merck, containing 0.2 % neutral sugars, 0.2 % amino sugars, and 0.3 – 0.5 % sialic acids [20]).

Dry weights. Protein samples were extensively dialyzed against twice-distilled water and dried to constant weight at 105°C. Weighing was performed in a Cahn microbalance.

Chemicals were of analytical grade.

Table 1. Amino acid recoveries after acid hydrolysis of *Pseudomonas* cytochrome *c* peroxidase. The results are given as grams of amino acid residues per 100 g of protein. The values for 20 h represent the mean of three separate determinations and those of 70 h that of two.

Amino acid residue	Time of hydrolysis		Average or extrapolated values
	20 h	70 h	
Aspartic acid	9.56	9.62	9.59
Threonine	3.67	3.60	3.70[a]
Serine	4.04	3.38	4.34[a]
Glutamic acid	11.75	11.75	11.75
Proline	6.19	6.21	6.20
Glycine	3.82	3.86	3.84
Alanine	6.16	6.12	6.14
Valine	5.42	5.53	5.53[b]
Methionine	(1.04)[c]	(0.47)[c]	–
Isoleucine	2.25	2.25	2.25
Leucine	8.03	7.96	8.00
Tyrosine	2.62	2.02	2.91[a]
Phenylalanine	6.17	6.34	6.34[b]
Lysine	6.71	6.74	6.73
Histidine	2.74	2.72	2.73
Arginine	4.66	4.72	4.69
Cystine	0	0	–

[a] Obtained by extrapolation to zero hydrolysis time. [b] 70 h value. [c] Sum of methionine and methionine sulfoxides.

RESULTS

Table 1 presents the amino acid recoveries from the samples of PsCCP hydrolyzed for 20 and 70 h. Decomposition with increasing hydrolysis time occurred with serine, tyrosine and, to a slight extent, threonine. The concentration of these amino acids was obtained by extrapolation to zero hydrolysis time by assuming first-order kinetics of destruction.[21] The yield of valine and phenylalanine was found to increase with increasing hydrolysis time. The values of the 70 h hydrolysates were used in the final calculation of these two amino acids. A tryptophan content of 1.65 % per unit weight of protein was obtained by analyzing colorimetrically the unhydrolyzed samples of PsCCP.[8] During the acid hydrolysis of PsCCP, cyst(e)ine is completely oxidized or

Table 2. Composition and molecular weight of *Pseudomonas* cytochrome *c* peroxidase.

Amino acid residue	Grams of amino acid residues per 100 g of protein[a]	Minimum molecular weight[b]	Amino acid residues per 43 200 g[c] of protein	Nearest integral No. of amino acid residues per 43 200 g of protein	Nearest integral No. multipl. by min. mol. weight
Aspartic acid	9.59	1 200	36.00	36	43 200
Threonine	3.70	2 732	15.81	16	43 712
Serine	4.34	2 006	21.54	22	44 132
Glutamic acid	11.75	1 099	39.31	39	42 861
Proline	6.20	1 566	27.59	28	43 848
Glycine	3.84	1 486	29.07	29	43 094
Alanine	6.14	1 157	37.34	37	42 809
Valine	5.53	1 793	24.09	24	43 032
Isoleucine	2.25	5 029	8.59	9	45 261
Leucine	8.00	1 414	30.55	31	43 834
Tyrosine	2.91	5 607	7.70	8	44 856
Phenylalanine	6.34	2 321	18.61	19	44 099
Lysine	6.73	1 904	22.69	23	43 792
Histidine	2.73	5 023	8.60	9	45 207
Arginine	4.69	3 330	12.97	13	43 290
Tryptophan	1.65[d]	11 300	3.83	4	45 200
Half cystine	0.65[e]	15 868	2.72	3	47 604
Methionine	1.22[e]	10 753	4.02	4	43 012
Amide ammonia	1.30[f]		35.10	35[f]	
	88.26				
Heme	2.85[g]				
Total	91.11[h]		350.80	354	44 047[i]

[a] From the last column of Table 1. [b] (Molecular weight of amino acid residue × 100)/percent amino acid residue in protein. [c] Molecular weight of PsCCP based on iron content.[3] [d] Determined by the method of Spies and Chambers.[8] [e] Determined after the performic acid oxidation according to Moore.[9] [f] Omitted from the total. [g] Calculated on the basis of iron content of PsCCP.[1,3] [h] Recovery on the basis of dry weight. The recovery of nitrogen calculated from the amino acid residues plus heme is 15.28 % nitrogen per unit weight of protein, which represents 97.4 % of the independently determined nitrogen content, 15.7 %. [i] Average molecular weight for all residues.

decomposed and methionine partially oxidized to methionine sulfoxides. Consequently, the sum of cysteine and cystine was determined as cysteic acid and methionine as methionine sulfone after performic acid oxidation.[9] Because no significant amounts of methionine, methionine sulfoxides or cystine were observed, the oxidation was considered to be complete. A cyst(e)ine content of 0.65 % and a methionine content of 1.22 % per unit weight of protein was so obtained. The amide ammonia analyses indicate the presence of 1.30 % amide residues per unit weight of protein. This corresponds to 35 amide groups per mol of PsCCP.

The nitrogen content of PsCCP calculated from the amino acid recoveries and including the nitrogen of heme groups is 15.28 %. An independent value of 15.7 % was obtained experimentally by the Dumas method.

No neutral sugars, amino sugars, or sialic acids were detected in carbohydrate analyses of PsCCP.

The composition and molecular weight of PsCCP is shown in Table 2. The accuracy with which the number of amino acids can be estimated depends on the number of residues present in the protein. The permitted variation would be $n \pm 0.4$ with n amino acid residues, and the accuracy therefore $\pm 0.4/n$.[22] The precision of the amino acid analyses may be estimated as better than 3 %, so that amino acid residues up to 13 per mol of protein can be calculated with reliability to the nearest integer. The total of amino acid residues in one molecule of the protein was estimated to be 354 and the average molecular weight, calculated from all amino acid residues, was found to be 44 047 with the standard deviation of 1212.

A value of 0.711 ml/g was calculated for the partial specific volume of PsCCP from the composition of the molecule using the weight percentages of

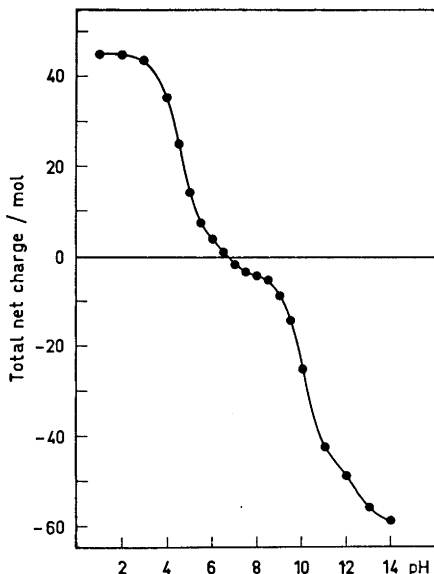

Fig. 1. A theoretical titration curve for *Pseudomonas* cytochrome *c* peroxidase. The calculation is based on the following pK values.[24,25]

Ionizable group	Total number in PsCCP	pK assumed
γ- and δ-Carboxyl	40	4.5
Imidazole	9	6.5
ε-Amino	23	10.0
Phenolic hydroxyl	19	10.0
Guanidine	13	12.5

The terminal amino and carboxyl groups and sulfhydryl groups assumed to be covalently bound to the heme groups were not considered. The titrateable carboxyl groups were obtained by subtracting the number of amide groups from the total of carboxyl groups.

the amino acid residues and their respective specific volumes [23] (heme groups omitted).

A theoretical titration curve for a total of 104 ionizable groups in the protein is shown in Fig. 1. For this hypothetical case an isoionic point of approximately 6.8 is obtained.

DISCUSSION

Acid hydrolysis of PsCCP was performed using the intact protein with the heme groups attached. The presence of iron in the hydrolysate may contribute to abnormal decomposition of amino acids during the hydrolysis. It is possible that the rate of decomposition of free amino acids may follow higher orders under these conditions.[26,27] Despite the addition of phenol to prevent the chlorination of tyrosine,[6] the loss of this amino acid during hydrolysis was considerable. No cystine and only traces of cysteic acid were detected in the chromatograms of the unoxidized hydrolysate; and methionine was partially oxidized to sulfoxides.

A considerable proportion of the acidic amino acid residues of PsCCP is in the amidated form (35 residues out of 75, the sum of aspartic acid and glutamic acid). Although the enzyme is soluble in aqueous solution at neutral pH, it contains an excess of hydrophobic amino acids (188 hydrophobic residues against 166 hydrophilic ones). The tyrosine and tryptophan as well as the cysteine and methionine content of PsCCP is quite low. Surprisingly, only three half cystines were recovered (if no unexpected losses of cysteine had occurred in the presence of iron) in a protein which, according to iron and heme analysis, contains two groups of heme *c* per molecule.[1,3] In C-type cytochromes there are generally two cysteine residues covalently linked with the vinyl groups of one protoheme; however, some exceptions are known. The hemopeptide of *Chromatium* cytochrome *cc'* contains two heme groups and three cysteine residues.[28] One heme is bound to two cysteine residues and the other, as deduced from the amino acid sequence of the hemopeptide, to one cysteine and presumably a threonine residue by an oxyether linkage.[28] This cytochrome yields a normal alkaline pyridine ferrohemochrome of heme *c* with an α-band at 551 nm.[29] Two other cytochromes containing one cysteine residue per heme are *Crithidia* cytochrome *c* [30] and *Euglena* cytochrome *c*.[31] These cytochromes, however, yield an alkaline pyridine ferrohemochrome with an α-band at 553 nm, thus differing from *Chromatium* cytochrome *cc'*. The linkages of the heme groups to the protein moiety in PsCCP require further study.

No neutral sugars, amino sugars, or sialic acids were detected in PsCCP. It thus differs in this respect too from plant peroxidases and lactoperoxidase which contain carbohydrates (neutral sugars and amino sugars) up to about 20 % of the dry weight.[18,32,33] PsCCP is similar to yeast cytochrome *c* peroxidase in containing no carbohydrates.[34]

The total recovery of amino acids on a dry weight basis was somewhat low. The recovery of nitrogen was, however, satisfactory. The low recovery on a dry weight basis may indicate the presence of some non-nitrogeneous component (not carbohydrate) in PsCCP, representing less than 6 % of dry weight.

The molecular weight, about 44 000, obtained from amino acid analyses, corresponds to the values based on the iron and heme analyses, 43 200 and 48 800, respectively.[3] The partial specific volume calculated from the chemical composition of PsCCP, 0.711 ml/g, is somewhat higher than the experimental value of 0.695 ml/g.[3]

The isoionic point, 6.8, obtained from the theoretical titration curve based on the amino acid composition of PsCCP, agrees well with the experimentally determined isoelectric point, 6.7.[1]

Acknowledgement. Automated nitrogen analyses were performed in the Biotechnical Laboratory of the Technical Research Centre of Finland, Helsinki, with the kind permission of Prof. T. M. Enari.

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Received February 14, 1973.