Amino acid composition of histidase

	μmole in acid hydrolysate (obtained from mole residue values as amount present in hydrolyzed samples) 48 h	72 h	mole residue/ mole enzyme for *P. putida* NCIB 10807	mole residue/ mole enzyme for *P. testosteroni* NCIB 10808[2]
Aspartic acid	0.1792	0.1864	182	164
Threonine	0.0621	0.0612	63	79
Serine	0.0740	0.0715	76	122
Glutamic acid	0.2014	0.2026	198	195
Proline	0.0753	0.0672	71	80
Glycine	0.1661	0.1620	164	159
Alanine	0.2675	0.02800	273	274
Valine	0.1361	0.1481	145	147
Methionine	0.0088	(0.0330)	22	26
Isoleucine	0.0952	0.0920	93	93
Leucine	0.2581	0.2453	251	224
Tyrosine	0.0245	0.0327	27	24
Phenylalanine	0.0542	0.0522	53	36
Lysine	0.0752	0.0790	76	43
Histidine	0.0485	0.493	49	46
Arginine	0.1410	0.0970	126	85
Cysteine	0.0028	(0.0027)	28	16

* The mol.wt of the enzyme is 200,000 and the calculations were based on this value.

0.17 ml absolute methanol cooled to 10 °C. The mixture was maintained at this temperature for 2 h and then an excess of water (10–15 ml) was added to terminate the reaction. The solution was freeze-dried and the sample hydrolyzed with 6 M-HCl as described above. Quantitative amino acid analysis was carried out by using an automatic analyser (Beckman Unichrome amino acid analyser with a high sensitivity flow cell).

Results and discussion. The amino acid composition of histidine ammonia-lyase from *P. putida* NCIB 10807 is presented (table). When the amino acid composition of *P. putida* NCIB 10807 is compared with that of *P. testosteroni,* the former has more basic amino acid residues, lysine and arginine than the latter. The cysteine content in *P. putida* enzyme tends to be higher than that of *P. testosteroni* enzyme. The variations in the amino acid compositions of the 2 enzymes may play a part in the ability of the enzyme to have multiple polymers or not. It is significant to note that the two histidine ammonia-lyases are not identical immunologically[10]. It is reasonable to suggest the absence of homologous regions between the 2 enzymes. Taxonomically the *Pseudomonas* are nutritionally diverse, and the ability to form pigment is variable. *P. putida* NCIB 10807 belongs to the fluorescent group, while *P. testosteroni* NCIB 10808 belongs to the nonfluorescent group. The observed variations in amino acid composition and the possibility of nonhomologous regions in the amino acid sequences of the histidine ammonia-lyases from the two organisms further separate the 2 organisms in terms of evolution of histidine ammonia-lyase in *P. putida* NCIB 10807 and *P. testosteroni* NCIB 10808.

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Chloroperoxidase-catalyzed oxidation of N-methyl-4-chloroaniline[1]

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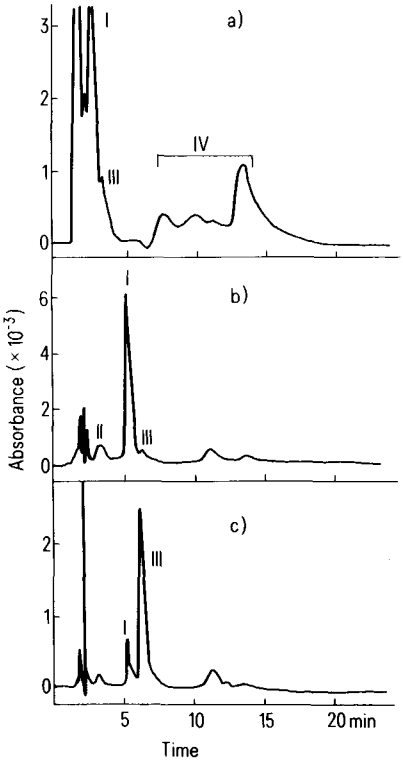
Summary. Chloroperoxidase catalyzed the H_2O_2 oxidative conversion of N-methyl-4-chloroaniline to 4-chloronitrosobenzene, 4-chloroaniline and a mixture of complex products.

We recently reported the facile oxidation of primary arylamines to nitrosoaromatics by hydrogen peroxide in the presence of chloroperoxidase[2]. This conversion is unique in that it is the only known enzymatic redox process that gives the nitroso oxidation state as the major product. We now report that chloroperoxidase also oxidizes secondary arylamines, and that a significant product of this conversion is the corresponding nitrosoaromatic. This study was conducted with N-methyl-4-chloroaniline as the model substrate for N-methyl arylamines.

Experimental. A solution of chloroperoxidase (Sigma Chemical Co.) consisting of approximately 9.6 μg/ml of protein in 0.05 M, pH 4.4 phosphate buffer was assayed for enzymatic activity according to the standard method[3]. In

the present study the chloroperoxidase solution contained 3.1 units/ml. A 2nd solution was prepared which contained the substrate and H_2O_2 at twice their desired final reaction concentrations in the same buffer. Studies were conducted with peroxide concentrations of 2 and 4 mM, and with substrate concentrations ranging from 0.125 mM to 2.0 mM. After equilibration of each solution to 25 °C, the reaction was initiated by combining equal volumes of the 2 solutions. The course of the reaction was then followed by monitoring for the production of 4-chloronitrosobenzene as measured by the increase in optical absorbance at 320 nm in a Beckman Model 24 spectrophotometer. The initial rate of reaction was taken to be that amount of nitroso product produced within the first 10 sec of the reaction. It had been previously established that within this 10 sec reaction time the rate of product formation was linear for all substrate concentrations investigated at both 2 and 4 mM peroxide.

A further analysis of the course of this enzymatic oxidation was made by employing high pressure liquid chromatographic (HPLC) techniques. Aliquots of each reaction were taken at appropriate timed intervals, and either injected directly onto the chromatographic column or quenched by adding an equal volume of ice-cold methanol. Samples taken by the latter method were kept at −20 °C and analyzed by HPLC within 2 h. The HPLC system utilized a C-18 μBondapak column (Waters Associates) with isocratic elution with 3 different solvent systems (figure) at a flow rate of 1.5 ml/min. Detection of the nitroso product was achieved by use of the 313 nm detector available on the Waters Model 440 detector. Detection of substrate and other reaction intermediates was achieved by use of the 254 nm detector. Sample injection size was usually 10 μl, and signal attenuation was adjusted according to which peak was being measured. Quantitation was achived by measurement of peak height, since preliminary experiments demonstrated a linear relationship between peak height and amount of authentic standards injected.



Representative HPLC analyses of the products resulting from chloroperoxidase-catalyzed oxidation of N-methyl-4-chloroaniline. The enzyme incubation contained 1.0 μmole of N-methyl-4-chloroaniline, 4.0 μmoles of H_2O_2 and 3.1 units of chloroperoxidase in 2.0 ml of 0.05 M, pH 4.4 phosphate buffer. The reaction was quenched by combining with an equal volume of MeOH. Analyses were conducted by injection of 10 μl aliquots of the quenched reaction onto a C-18 μBondapak column followed by elution at a flow rate of 1.5 ml/min under the following conditions: *a* 2-Propanol/MeOH/H_2O (2:6:2) with detection at 254 nm; *b* 65% MeOH with detection at 254 nm; *c* 65% MeOH with detection at 313 nm. Peak assignments: I: N-methyl-4-chloroaniline, II: 4-chloroaniline, III: 4-chloronitrosobenzene, IV: mixture of complex oxidation products.

Results and discussion. We found that the chloroperoxidase-catalyzed oxidation of N-methyl-4-chloroaniline proceeded rapidly to give 4-chloronitrosobenzene as a major product. Trace amounts of 4-chloroaniline were also observed. Unlike our previous discovery that the nitroso compound is the only significant product resulting from chloroperoxidase-catalyzed oxidation of 4-chloroaniline[2], the present reaction obviously gave additional more complex products (figure). The nature of these products and the mechanisms by which they are formed is not yet known, although they are possibly similar to the products formed by horseradish peroxidase oxidation of anilines[4].

The kinetics of this oxidative process could not be followed by a measurement of the rate of disappearance of the substrate. However, the rate of formation of the nitroso compound did display typical enzyme saturation kinetics, and indicated a relative K_m of 0.9 mM in the presence of 4 mM peroxide.

The percent conversion of the substrate to 4-chloronitrosobenzene varied from 2 to 8% over the substrate concentration range investigated. The highest percent conversion to nitroso compound occurred at the lowest substrate concentration. HPLC analysis indicated that the maximum formation of nitroso compound occurred after about 5 min reaction time, and that the level of this product remained constant after that time. HPLC analysis also demonstrated the presence of 4-chloroaniline in the reaction product at all times from 30 sec to 10 min incubation time.

The maximum observed percent conversion of substrate to 4-chloroaniline was 2%, which was found at the highest substrate concentration employed, while an 0.8% conversion was found at the lowest substrate concentration. Typical HPLC analyses of reaction products are indicated in the figure.

Although the oxidation of N-methyl-4-chloroaniline by H_2O_2 in the presence of chloroperoxidase is obviously more complex than is the oxidation of 4-chloroaniline[2], we feel that the present results indicate that chloroperoxidase can effect a demethylation of N-methylanilines. The oxidative conversion of the N-methyl aniline to an aniline, followed by the known conversion of primary anilines to nitrosoaromatics explains part of the mechanism of this oxidation by chloroperoxidase. In view of our previous results on the oxidation of 4-chloroaniline[2], it is probable that the pathway for the formation of the complex oxidation products from N-methyl-4-chloroaniline does not proceed through the intermediate formation of 4-chloroaniline.

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