Possible Active Sites in Nitrite Reductase and Hydroxylamine Reductases from Vegetable Marrow (*Cucurbita pepo* L.)

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In plants, nitrite reductase behaves as a single protein producing NH_3 without involving free hydroxylamine and is dependent on one-electron donors: reduced benzyl viologen or ferredoxin (see Hewitt, Hucklesby & Betts, 1968). A closely associated hydroxylamine reductase (hydroxylamine reductase 1) utilizes additionally FMNH_2 , FADH_2 (Hewitt & Hucklesby, 1966; Hewitt *et al.* 1968) and NADPH_2 . Another hydroxylamine reductase (hydroxylamine reductase 2) functions at present only with reduced benzyl viologen or dithionite. In fungi (see Nicholas, 1959) flavoproteins containing iron plus copper in nitrite reductase and manganese in hydroxylamine reductase occur. In soya bean nitrite reductase and hydroxylamine reductase were thought to be flavoproteins activated by manganese (Nason, Abraham & Averbach, 1954). Experiments with azide, 1,1'-bipyridyl, *o*-phenanthroline, 8-hydroxyquinoline, diethyldithiocarbamate or EDTA did not indicate metals in nitrite reductase or hydroxylamine reductase from marrow (Cresswell, Hageman, Hewitt & Hucklesby, 1965), but cyanide (pH 7.5) inhibited nitrite reductase by 85% at 0.1 mM and hydroxylamine reductase by 55% at 0.2 mM, with reduced benzyl viologen as electron donor.

CO inhibits nitrite reductase and hydroxylamine reductase 1 (reduced benzyl viologen as electron donor) irreversibly in light. Inhibition of hydroxylamine reductase 2 by CO is reversed by light. Activity of hydroxylamine reductase 2 is related to light-absorption at 399 nm. This peak shifts to 415 or 433 nm on reduction by reduced benzyl viologen or dithionite respectively and returns to 399 nm on autooxidation. Pyridine, dithionite and 0.1 M-NaOH induce peaks at 419, 524 and 557 nm. CO with dithionite produces a sharper peak at 420 nm. Comparable nitrite reductase preparations do not show these peaks.

The iron chelator bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) disulphonate inhibits nitrite reductase by 50% at 0.1 mM and hydroxylamine reductase 2 by 50% at 1 mM, with reduced benzyl viologen as electron donor and 0.3 mM substrate. Iron reverses the inhibition of nitrite reductase.

The copper chelators 2,2'-biquinolyl, 2,9-dimethyl-1,10-phenanthroline, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline and biscyclohexanoneoxalyl dihydrazone at saturation and antimycin A and 2-*n*-

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heptyl-4-hydroxyquinoline *N*-oxide (400 $\mu\text{g}/\text{mg}$ of protein) did not inhibit nitrite reductase or either of the hydroxylamine reductases appreciably.

Phenylhydrazine and semicarbazide (10 mM) did not inhibit nitrate reductase or hydroxylamine reductase 1, but semicarbazide, hydrazine and isonicotinic acid hydrazide (5–50 mM) inhibited hydroxylamine reductase 2 by 20–36%. A flavin component in nitrite reductase (Huzisige, Satch, Tanaka & Hyashida, 1963) was not confirmed by precipitation with acid $(\text{NH}_4)_2\text{SO}_4$. Weak unpredictable nitrite reductase activity may occur with FMNH_2 , and hydroxylamine reductase 1 is inhibited by 25–100% by 10 mM-atabrine with FMNH_2 as electron donor.

Both nitrite reductase-hydroxylamine reductase 1 and hydroxylamine reductase 2 may be iron-containing proteins with possibly haem and carbonyl groups in the latter.

- Cresswell, C. F., Hageman, R. H., Hewitt, E. J. & Hucklesby, D. P. (1965). *Biochem. J.* **94**, 40.
 Hewitt, E. J. & Hucklesby, D. P. (1966). *Biochem. biophys. Res. Commun.* **25**, 689.
 Hewitt, E. J., Hucklesby, D. P. & Betts, G. F. (1968). In *Recent Aspects of Nitrogen Metabolism in Plants*, p. 47. Ed. by Hewitt, E. J. & Cutting, C. V. London and New York: Academic Press.
 Huzisige, H., Satch, K., Tanaka, K. & Hyashida, T. (1963). *Pl. Cell Physiol.*, Tokyo, **4**, 307.
 Nason, A., Abraham, R. G. & Averbach, B. D. (1954). *Biochim. biophys. Acta*, **15**, 159.
 Nicholas, D. J. D. (1959). *Symp. Soc. exp. Biol.* **13**, 1.

Bovine Brain Creatine Phosphotransferase: Amino Acid Sequence around the Essential Thiol Groups

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The guanidino phosphotransferases form a closely related group of enzymes of similar properties and function. When creatine phosphotransferases from muscle and brain tissues of the same species are compared significant differences in their K_m values, stability, response to alkylation, amino acid composition and peptide 'maps' are observed (Eppenberger, Dawson & Kaplan, 1967; Dawson, Eppenberger & Kaplan, 1967; Thomson, Eveleigh, Laws & Miles, 1968; Atherton & Thomson, 1969). However, there is good evidence that in the region of their essential thiol groups the amino acid sequences are similar (Thomson *et al.* 1968). This has been investigated further.

Pure bovine brain creatine phosphotransferase was

labelled with iodo[2-¹⁴C]acetate and digested with trypsin (Thomson, Eveleigh & Miles, 1964). The resulting peptides were purified by ion-exchange and gel-filtration chromatography and by high-voltage electrophoresis. Chymotryptic digests of the major radioactive tryptic peptides were similarly treated. The amino acid sequences of peptides were determined by the 'dansyl'-Edman method (Gray, 1967).

From the sequences obtained, together with amino acid analysis data, it was deduced that the amino acid sequence around the reactive thiol groups of bovine brain creatine phosphotransferase is:

Asx-Tyr-Glx-Phe-Met(Glx,Asx,His,Leu,Gly,Tyr)Ile-Leu-Thr-CMC-Pro-Ser-Asx-Leu-Gly-Thr-Gly-Leu-Arg

where CMC represents *S*-carboxymethylcysteine.

The sequence from isoleucine to arginine is identical with that in the enzymes from rabbit and bovine muscle (Thomson *et al.* 1964, 1968) except that isoleucine substitutes for valine. Arginine phosphotransferase from lobster has a very similar sequence (Thoai, 1968). Further, the sequence from phenylalanine to arginine (except for isoleucine) is likely to be identical with the corresponding sequence in muscle creatine phosphotransferases. The yields of labelled peptides together with kinetic evidence suggests that the chemical environments of the two essential thiol groups of the brain enzyme are identical.

It is confirmed that, despite differences in primary structure in other parts of the molecule, the sequence around the reactive thiol groups of bovine brain creatine phosphotransferase is virtually identical with that of muscle enzyme. This part of the sequence of guanidino phosphotransferases has been substantially conserved throughout their evolution. These enzymes should thus prove invaluable in unravelling evolutionary pathways.

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- Atherton, R. S. & Thomson, A. R. (1969). *Biochem. J.* **111**, 797.
- Dawson, D. M., Eppenberger, H. M. & Kaplan, N. O. (1967). *J. biol. Chem.* **242**, 210.
- Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967). *J. biol. Chem.* **242**, 204.
- Gray, W. R. (1967). In *Methods of Enzymology*, vol. 11, p. 139. Ed. by Hirs, C. H. W. New York: Academic Press Inc.
- Thoai, N. V. (1969). *Abstr. FEBS 6th Meet., Madrid*, no. 66.
- Thomson, A. R., Eveleigh, J. W., Laws, J. F. & Miles, B. J. (1968). In *Biochemical Evolution, and Homologous Enzymes*, p. 255. Ed. by Thoai, N. V. & Roche, J. New York: Gordon and Breach.
- Thomson, A. R., Eveleigh, J. W. & Miles, B. J. (1964). *Nature, Lond.*, **203**, 267.

Study by Mass Spectrometry of Amino Acid Sequences in Peptides containing Histidine

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The choice of a method for the preparation of peptide derivatives suitable for mass spectrometry depends to a certain extent on the composition of the peptides. It is now common practice, besides blocking of the functional groups, to permethylate the peptide bonds. The permethylation of peptides containing histidine, however, gives rise to some problems.

Our procedure is the following. We prepare a protected peptide by dissolution of 0.01 mmol of a peptide in 2 ml of water and subsequently add 0.01 mmol of diethyl pyrocarbonate/acylatable group. The pH is kept constant at 8.0 with a pH-stat for 30 min. After acidification with 1 M-HCl the ethoxycarbonylated component is extracted with ethyl acetate (Kamerling, Heerma & Vliegenthart, 1968). Besides the amino group, the phenolic hydroxyl group of tyrosine, the thiol group of cysteine and the *N*-imidazole group of histidine are also ethoxycarbonylated. The peptide derivative is subsequently permethylated with methyl iodide and methylsulphonyl carbanion as base in dimethyl sulphoxide (Vilkas & Lederer, 1968). The application of this method to histidine-containing peptides leads to the formation of a quaternary base, which remains in the water phase during the extraction with chloroform. We were able to circumvent this problem by cleavage of the imidazole ring of histidine with an excess of diethyl pyrocarbonate. After the reaction of the peptide with the initial amount of diethyl pyrocarbonate an excess of 4 mol of reagent/mol of histidine residue is added in four 1 mol portions at 30 min intervals at constant pH 8.0. This operation splits off the C-2 atom of the imidazole ring as formate, and converts the remaining part of the ring into the 1,2-bis(ethoxycarbonylamino)ethylene group. After acidification the reaction product can be isolated by extraction with ethyl acetate and subsequently be permethylated. The product is now soluble in chloroform and suitable for mass spectrometry.

Histidine itself can also be cleaved by exhaustive treatment with diethyl pyrocarbonate at pH 8.0, yielding 2,4,5-tris(ethoxycarbonylamino)pent-4-enoic acid. This product contains a small percentage of bis(ethoxycarbonyl)histidine. The cleavage reaction is comparable with cleavage of the imidazole ring in histidine methyl ester with benzoyl chloride under Schotten-Baumann conditions (Ashley & Harington, 1930). After permethylation of 2,4,5-tris(ethoxycarbonylamino)pent-4-enoic acid, the mass spectrum showed the presence of exclusively