Lack of distant relationships between lysozyme Ch and hen egg white lysozyme: computer comparison studies[1]

Jin-Jyi Chang and J. H. Hash[2]

Department of Microbiology, Vanderbilt University School of Medicine, Nashville (TN 37232, USA), 13 August 1980

Summary. A computer search, made for distant relationships between lysozyme Ch, hen egg white lysozyme, and bacteriophage T4 lysozyme, revealed no unusual similarities in their amino acid sequences. Also, antibodies generated against lysozyme Ch failed to cross react with hen egg white lysozyme and vice versa. These lysozymes most likely represent examples of convergent evolution.

Lysozymes are ubiquitous enzymes, and 5 distinct types have been characterized. The most intensively investigated and consequently the best understood are those from avian egg white. There are 2 distinct types of enzyme from this source: hen egg white lysozyme (E.C. 3.2.1.17, HEWL, lysozyme c)[3] and goose egg white lysozyme (lysozyme g)[4]. Other distinct types of lysozyme are produced by bacteriophages[5], plants[6], and invertebrates[7]. It is now apparent that lysozyme Ch from the fungus, *Chalaropsis* species, recently sequenced by this laboratory[8], represents yet a 6th distinct type of lysozyme.

HEWL is a β-1,4-N-acetylmuramidase, cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine in the bacterial cell wall matrix[9]. If the N-acetylmuramic acid is 6-0-acetylated, the cell wall is insensitive to the action of HEWL. This acetylation represents one of the mechanisms of resistance of certain strains of bacteria to HEWL[10]. The reason for this insensitivity did not become clear until the 3-dimensional structure of HEWL was solved by X-ray crystallography[11]. HEWL is characterized by a cleft in the molecule into which the linear strands of the cell wall substrate fit for catalysis. If the 6-hydroxyl group of N-acetylmuramic acid is acetylated, steric constraints prevent the substrate from fitting into this cleft and hence the cell wall is resistant to lysozyme action. The amino acids lining the cleft are responsible for the binding and alignment of the substrate as well as catalysis: the 2 most important for catalysis are glutamic acid-35 and aspartic acid-52[12].

Part of the interest in lysozyme CH stems from the fact that, in addition to possessing β-1,4-N-acetylmuramidase activity identical to HEWL, it also possesses β-1,4-N,6-0-diacetylmuramidase activity. Thus, lysozyme Ch is active against bacterial species that are insensitive to HEWL by virtue of being 6-0-acetylated. Presumably, the cleft of lysozyme Ch is different from that of HEWL in that it can accommodate a bulky acetyl group without steric hindrance. Visual inspection of the sequence of lysozyme Ch revealed no apparent homologies with the following lysozymes whose sequences are known: hen egg white, guinea-hen egg white, turkey egg white, duck egg white, Japanese quail egg white, T2 phage, T4 phage, baboon milk, and human[8]. We have recently established that the catalytic activity of lysozyme Ch is dependent on two acidic residues: aspartic acid-6 and glutamic acid-33[13].

It was therefore of some interest to examine lysozyme Ch and HEWL for possible phylogenetic relationships. The methods selected were immunological cross reactivity and a computer program designed to detect distant relationships between protein sequences.

Materials and methods. The computer program ALIGN and RELATE[14], using both a unitary and a mutation data matrix[15], are designed to detect very distant relationships between protein sequences and also, internal duplication within proteins. Bacteriophage T4 lysozyme was included in the comparison. The length of fragments compared between lysozyme Ch, HEWL, and T4 was 20 residues, and when compared with itself, 10 residues. In all cases, the number of random runs per comparison was 100.

Results and discussion. The segment comparison scores of the lysozymes with the program RELATE are given in the table. All of the scores, from either matrix, are not significantly different from scores expected from a comparison of random sequences. There is no evidence for gene duplication, as evidenced by the segment comparison score of 0.3, when lysozyme Ch was compared with itself. The program ALIGN gave a low alignment score of 1.26 between lysozyme Ch and HEWL, indicating no relationship between the two.

As expected, antibodies against lysozyme Ch and egg white lysozyme, generated in rabbits, showed no cross-reactivity in the Ouchterlony[16] double diffusion technique.

It is concluded that the computer comparison of sequence data and the lack of immunological cross-reactivity support the view that lysozyme Ch and HEWL are 2 structurally unrelated proteins that arose independently in the past. Sequence changes in proteins are considered to have occurred by 3 evolutionary mechanisms that are not mutually exclusive: divergent, convergent, and parallel[17]. Divergent evolution is considered to be the result of a common ancestral gene that underwent mutations and amino acid changes not essential to function were permissable and the proteins 'diverged' in sequence (e.g., hemoglobins and cytochromes). Convergent evolution is considered to be the independent evolution of 2 dissimilar proteins to perform identical functions via the same mechanism. In this case, the function 'converged' (e.g., subtilisin and pancreatic serine proteases). Parallel evolution is considered to be the independent evolution of dissimilar proteins with similar functions but with a different mechanistic basis for their activity (e.g., trypsin, pepsin and papain).

It is concluded that, because lysozyme Ch and HEWL are dissimilar in sequences but use glutamic acid and aspartic acid in their catalytic mechanisms, these 2 lysozymes most likely represent examples of convergent evolution. As lysozymes from other sources are characterized it will be of interest to determine whether alternative mechanisms involving different amino acid residues have evolved for the catalytic cleavage of the bacterial cell wall peptidoglycan.

Segment comparison scores of lysozyme Ch with HEWL and T4 lysozyme (in SD units)

	HEWL	T4	Ch
	Unitary matrix		
Lysozyme Ch	−0.8	1.4	0.3
	Mutation data matrix		
Lysozyme Ch	3.0	0.2	-

1 Acknowledgments. This research was supported by Public Health Service Grant AI-15531 and the Biomedical Research Support Grant RR-05424.

2 To whom all communications should be addressed.

3 R.E. Canfield, J. biol. Chem. *238*, 2698 (1963).

4 R.E. Canfield, S. Kammerman, J.H. Sobel and F.J. Morgan, Nature New Biol. *232*, 16 (1971).

5 A. Tsugita and M. Inouye, J. molec. Biol. *37*, 201 (1968).

6 J.B. Howard and A.N. Glazer, J. biol. Chem. *244*, 1399 (1969).
7 J. Jollès and P. Jollès, Eur. J. Biochem. *54*, 19 (1975).
8 J.W. Felch, T. Inagami and J.H. Hash, J. biol. Chem. *250*, 3713 (1975).
9 D.M. Chipman and N. Sharon, Science *165*, 454 (1969).
10 J.-M. Ghuysen and J.L. Strominger, Biochemistry *2*, 1119 (1963).
11 D.C. Phillips, Proc. natl Acad. Sci. USA *57*, 484 (1967).
12 T.-Y. Lin and D.E. Koshland, Jr, J. biol. Chem. *244*, 505 (1969).
13 P.B. Fouche and J.H. Hash, J. biol. Chem. *253*, 6787 (1978).
14 M.O. Dayhoff, in: Atlas of Protein Sequence and Structure, vol.5, suppl.2, p.1. Ed. M.O. Dayhoff. National Biomedical Research Foundation, Washington, DC 1976.
15 W.C. Barker and M.O. Dayhoff, in: Atlas of Protein Sequence and Structure, vol.5, p.101. Ed. M.O. Dayhoff. National Biomedical Research Foundation, Washington, DC 1972.
16 O. Ouchterlony, Acta path. microbiol. scand. *26*, 507 (1949).
17 E.L. Smith, in: The enzymes, vol.1, p.267. Ed. P.D. Boyer. Academic Press, New York 1970.

The antifertility action of α-chlorohydrin: metabolism by rat and boar sperm[1]

A.R. Jones, Denise Stevenson, Primrose Hutton and A.G. Dawson[2]

Department of Biochemistry, University of Sydney, Sydney (N.S.W. 2006, Australia), 2 September 1980

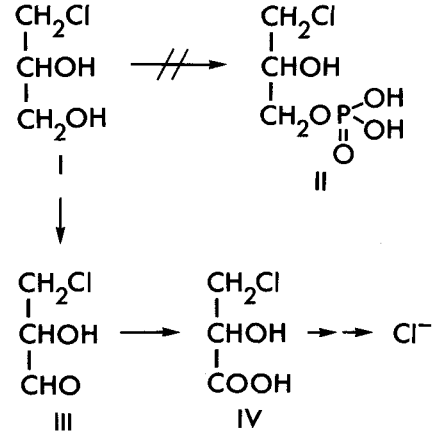
Summary. The male antifertility agent α-chlorohydrin (**I**) is metabolized by rat and boar sperm to β-chloroactaldehyde (**III**), β-chlorolactate (**IV**) and Cl^- and not to the proposed active metabolite, α-chlorohydrin-l-phosphate (**III**). It is proposed that β-chlorolactaldehyde is produced intracellularly by a specific enzyme and that this is the metabolite responsible for the species-specific antifertility activity of α-chlorohydrin.

The current theory[3] for the mechanism of action of α-chlorohydrin (3-chloropropan-1,2-diol, **I**) as a male antifertility agent is that, upon entry into sperm, it is converted by glycerol kinase to α-chlorohydrin-l-phosphate (**II**). This metabolite is proposed to inhibit glyceraldehyde-3-phosphate dehydrogenase and triosephosphate isomerase thereby inhibiting glycolysis and resulting in a decreased production of ATP. When ejaculated, the sperm are morphologically normal but have such diminished motility that fertilization can not be achieved.

Our studies into the metabolism of α-chlorohydrin by rat and boar sperm have led us to question this hypothesis, for when sperm were incubated with ^{36}Cl-α-chlorohydrin, ^{36}Cl-α-chlorohydrin-l-phosphate[4] could not be detected. Furthermore, glycerol kinase[5] was found to be unreactive towards α-chlorohydrin, a result which has recently been confirmed by Brooks[6]. It is known that α-chlorohydrin per se is not the inhibitory agent of sperm glycolysis as a period of pre-incubation is required before an inhibitory effect is evident in vitro[7]. On the basis that this indicates the production of an active metabolite of α-chlorohydrin, we have investigated the comparative metabolisms of ^{36}Cl-α-chlorohydrin by rat, boar and rabbit sperm, and by isolated kidney tubules of each species.

Results. Sperm obtained from the cauda epididymides of mature boars within 1.5 h, or adult rats or rabbits within 0.5 h of sacrifice, were washed with phosphate-buffered saline[8] (PBS) (pH 7.4) and centrifuged. This washing procedure was repeated twice. In a typical experiment, a suspension of rat or boar sperm in PBS (5 ml, containing 20–40 mg protein ml^{-1}) was incubated at 34 °C with 1 mM fructose and 100 μM ^{36}Cl-α-chlorohydrin[9] for 2 h, then centrifuged. When assayed by TLC (silica gel G plates developed in chloroform:methanol 7:3 followed by radiochromatogram scanning), the supernatant was found to contain ^{36}Cl-α-chlorohydrin (R_f 0.55) and trace amounts of $^{36}Cl^-$ (< 5%) as the only radioactive constituents. The pellet was suspended in PBS (1 ml), the cells disrupted by sonic oscillation (20 kHz for 2 min) and centrifuged. When this supernatant was examined by TLC, 4 radioactive components were detected. Three of these were identified as described previously[10]; they were Cl^- (R_f 0.10), β-chlorolactate (**IV**) (R_f 0.25) and α-chlorohydrin. The 4th component had a polarity between the latter 2 compounds at R_f 0.47 and was base labile, indicating that it possessed the α-halohydrin structure. When the supernatant was treated with 2,4-dinitrophenylhydrazine (2,4-DNP) reagent[11], extracted with ethyl acetate and the extract examined by TLC, the compound at R_f 0.47 was absent but was replaced by a ^{36}Cl-2,4-DNP derivative at R_f 0.66, corresponding to authentic[10] β-chlorolactaldehyde-2,4-DNP. There was no metabolism of α-chlorohydrin by rabbit sperm or by suspensions of isolated kidney tubules of the boar, rat or rabbit.

Glycolytic inhibition studies were performed in conventional Warburg flasks with 1 ml cell suspensions containing D-[U-^{14}C]fructose (1 mM) and α-chlorohydrin (10 mM). Sperm suspensions (20–40 mg protein ml^{-1}) or isolated kidney tubules[12] (4–15 mg protein ml^{-1}) were incubated at 34 °C and 37 °C, respectively, for 1 h and the $^{14}CO_2$, trapped in 2 M NaOH, was assayed by established procedures[8]. The inhibition of $^{14}CO_2$ production was found to be 91% with boar sperm and 67% with rat sperm. There was no effect on



The metabolism of α-chlorohydrin (**I**) in rat and boar sperm. The phosphorylated derivative (**II**) is not produced, instead oxidation gives β-chlorolactaldehyde (**III**), β-chlorolactate (**IV**) and Cl^-.