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Publication Date
1988-11-01

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November 1988
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Mechanistic and Evolutionary Studies on Genetically Engineered Chicken Lysozyme

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Chicken egg-white lysozyme (HEL) has served as a paradigm for a number of sub-fields of biochemistry in addition to immunology. We are concerned here with applications of the cloned site-directed mutagenized enzyme to mechanistic enzymology and to biochemical evolution.

Mutagenesis and Expression

The cloned c-DNA was mutagenized in E. coli and expressed as a secreted protein in yeast as described elsewhere. Typical yields are ca. 5 mg of HEL per liter of yeast culture medium.

The roles of Glu35 and Asp52 in Catalysis

The catalytic mechanism generally accepted for the mechanism of action of lysozyme was formulated almost entirely from an examination of the crystal structure. Residues GLU35 and ASP52 were proposed to act coordinately to generate an oxocarbocation at the sensitive glycosidic linkage at subsite D (Scheme 1). The γ carboxylic acid of GLU35 assists the expulsion of the leaving group by general-acid catalysis, while the developing oxocarbocation is stabilized by electrostatic interaction with ASP52. This proposed mechanism was supported by later work with secondary...
hydrogen$^3,^4$ and primary oxygen-18$^5$ kinetic isotope effects which provide further evidence for a transition state with nearly fully developed oxocarbocation character$^5$.

Some aspects of this mechanism were probed by examining the kinetic behavior of the mutants E35Q (glutamate replaced with glutamine at position 35) and D52N in various assays. The mutant E35Q shows no detectable activity with any substrate, while D52N has 5% activity in the M.-luteus cell-wall assay and none with the soluble substrates glycol chitin or chitopentaose. The 5% activity detectable in the cell-wall assay is biphasic, in that the rate of reaction diminishes after a short period of time to a constant level of ca. 0.5% of that of wild-type enzyme, suggesting that there may be a set of linkages in the heterogeneous mixture that is particularly susceptible to catalysis by the mutant protein. The affinities of the mutant protein for the inhibitor, chitotriose ((GlcNAc)$_3$), are within a factor of 2 of that of wild-type. These experiments are documented more fully in (1).

The Role of ASP101 in Ligand Association

The crystallographic results of Blake et al.$^2$ showed hydrogen bonds formed between the carboxylate of ASP101 and the acetamido proton of the GlcNAc moiety bound in the A subsite of enzyme. Another hydrogen bond was seen with the hydroxymethyl portion of the sugar moiety bound in the B subsite (Scheme I). This model accounts for the observation
that the affinity of chicken lysozyme for \((\text{GlcNAC})_3\) increases fourfold depending upon a pK\(_a\) of 4.2. Consistent with that model Arnheim et al. found that turkey lysozyme, which has glycine in position 101 replacing the aspartate of chicken, does not show the increased affinity dependent upon the pK\(_a\) of 4.2\(^6\). They therefore concluded that this ionization was due to ASP101.

Turkey lysozyme, however, has six additional amino-acid differences from chicken lysozyme, which makes the latter conclusion tenuous. The pH dependence of \(K_d\) for the \((\text{GlcNAC})_3\)-lysozyme complex of the D101G chicken lysozyme mutant does not exhibit the increase in affinity above pH 4 shown by the chicken wild-type enzyme \((\text{GlcNAC})_3\) complex. The ambiguity remaining from the earlier work is therefore removed, in that the pK\(_a\) of 4.2 can be cleanly assigned to ASP101. The fourfold increase in affinity due to the formation of this hydrogen bond in wild-type enzyme corresponds only to 0.8 kcal/mol, a figure much lower than the estimates of 3-6 kcal/mol resulting from Fersht's survey of hydrogen bond interactions between neutral and charged species\(^7\).

The amino acids SER, ASN, LYS, ARG, and GLU were also introduced individually into position 101, and the pH dependence of \(K_d\) for dissociation of the \((\text{GlcNAC})_3\)-HEL complexes were determined. The main results are that all of the mutants bearing neutral side chains in position 101 (ASN, SER and GLY) and unionized acidic side chains (ASP and GLU)
show approximately the same affinity for (GlcNAc)₃ at low pH. By contrast, the basic mutants D101R and D101K exhibit Kₐ values which are ca. twofold higher than those of the neutrals. The values of Kₐ increase approximately twofold for the neutral and cationic amino acids in position 101 upon raising the pH to 5.5. Wild-type enzyme has a Kₐ fourfold lower than the mutants bearing neutral amino-acid side chains in position 101. The behavior of D101E is intermediate between wild type and the neutrals, suggesting some lesser interaction between the glutamate side chain and (GlcNAc)₃.

_Coupled Changes during Molecular Evolution_

Evolutionary trees are commonly constructed, in part, from comparing amino-acid sequences of extant proteins. The most closely related species generally have the fewest sequence differences. Although the amino-acid sequences of ancestral proteins can frequently be inferred through such techniques as parsimony analysis, the direct study of the properties of proteins from extinct species has, with rare exceptions, been precluded. Site-directed mutagenesis now allows such reconstructions to be made, and thus opens new experimental vistas for the study of molecular evolution.

An example of the utility of such an approach emanates from an examination of a partial evolutionary tree of the gallinaceous birds. The amino acids in positions 40, 55 and 91 are in close proximity at the bottom of the active-site cleft of lysozyme. Chicken lysozyme and that of most other
present-day species have amino acids THR, ILE and SER, at positions 40, 55 and 91, respectively, while the Guinea fowl (Numida meleagris) and the New-World quails (Odontophorinae) have SER, VAL, and THR at these positions. No intermediate forms are known to occur naturally. Since the Guinea fowl diverged from the chicken line 35-40 million years ago, while the New-World quails probably split off from this lineage only 15-30 million years ago, this coordinate change in the three amino acids had to have occurred at least twice in the course of evolution. The probability of a triple mutation is infinitesimal; therefore, according to the most parsimonious pathway between the modern forms, at least two of the six intermediate forms shown in Scheme 2 must have existed for some period. One question to be asked is why they did not survive.

The genes for all six possible intermediates were constructed and expressed in yeast. All six of these synthetic lysozymes are active against a variety of substrates. More complete characterization of them will be described elsewhere (B. A. Malcolm et al., in preparation). Our attention was drawn to possible differences in thermal stability because of the significant variation in this parameter found by K. Olsen (unpublished results) between the lysozymes of chicken and New-World quails.

Thermostability and Molecular Volume
Significant variation in $T_m$'s do exist among the eight lysozymes (Table 1). The value of $T_m$ is highly correlated with the molecular volume of the amino acid side chains ($r = 0.96$). Each additional methyl or methylene added in one of these positions contributes an average of 2-3°C to the increase in $T_m$. In a related study, Matsumura et al.\textsuperscript{12} recently reported that substituting SER for THR and VAL for ILE in position three of bacteriophage T4 lysozyme reduces $T_m$ by 2.1 and 0.9°C, respectively. It would appear that considerable thermal stabilization of enzymes intended for production purposes might be achieved by the packing of hydrophobic residues in selected regions through protein engineering.

**Thermostability and Natural Selection**

The changes in $T_m$'s near 70°C are in a temperature range which might seem unlikely to have much effect upon lysozyme survival at the incubation temperature of 37 to 40°C\textsuperscript{13}; however, the observation by Cunningham and Lineweaver that ovalbumin, the most abundant protein in egg white, significantly lowers the thermal stability of lysozyme\textsuperscript{14} proved to be intriguing. The pH value of egg whites, which is near neutral at the time of laying, rises to slightly above 9 for a period of several days and then declines slowly to the neutral range by day 9, at which time the remaining egg white is subsumed into the amnion. Lysozyme is relatively thermolabile at pH 9; therefore measurements of the rate
constants for the inactivation of lysozyme were made at this pH as a function of temperature, as were separate determinations of the half-life of the protein at 37°C in the presence of ovalbumin. The extant chicken and quail isozymes both have half-lives under these conditions of 17 ± (2 or 3) days. The SIT and SIS variants, have half-lives of only 11 ± 1 and 12 ± 2 days, respectively. This factor may have been a selective constraint against their survival, and if so, would eliminate three of the six possible evolutionary pathways under consideration. It is clear that increased thermal stability under near in vivo conditions cannot be the only evolutionary driving force because the most stable putative intermediate, TIT, which has a half-life of 22 ± 5 days has not yet been observed among surviving species.

Supported by the U. S. Department of Energy under Contract DE-AC03-76SF00098.

References


Scheme 1. Schematic representation of the transition state in hexasaccharide-cleavage by chicken egg-white lysozyme. The extended binding site is shown, together with the scissile glycosidic bond and the catalytic residues E35 and D52.

Scheme 2. The six possible intermediates separating present day quail from chicken lysozymes are shown in outline along the vertices of the cube.
Table I: Variation of $T_m$ with Amino Acid Side Chain Volume for the Combined Lysozyme Positions 40, 55, and 91a

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Combined Side Chain Volume (Å³)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIS (chicken)</td>
<td>390</td>
<td>73.9 (0.1)</td>
</tr>
<tr>
<td>SIS</td>
<td>367</td>
<td>73.0 (0.1)</td>
</tr>
<tr>
<td>TVS</td>
<td>363</td>
<td>71.2 (0.1)</td>
</tr>
<tr>
<td>TIT</td>
<td>413</td>
<td>77.5 (0.1)</td>
</tr>
<tr>
<td>SVS</td>
<td>340</td>
<td>70.6 (0.6)</td>
</tr>
<tr>
<td>SIT</td>
<td>390</td>
<td>75.5 (0.1)</td>
</tr>
<tr>
<td>TVT</td>
<td>386</td>
<td>74.5 (0.2)</td>
</tr>
<tr>
<td>SVT (quail)</td>
<td>363</td>
<td>73.4 (0.1)</td>
</tr>
</tbody>
</table>
The extent of denaturation was monitored spectrophotometrically at 292nm. Reactions were carried out in 100mM potassium phosphate buffers at pH 6.3.
Scheme 1
Scheme 2
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