ON THE IDENTITY OF SUBTILISINS BPN' AND NOVO

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Summary: A 2.5 Å difference-electron-density map between subtilisins BPN' and Novo contains no features larger than approximately one-quarter of a water molecule. It is therefore very likely that the two enzymes obtained from different commercial sources are identical, confirming results of peptide mapping experiments (1). Most of the small peaks and holes in the map lie close to the molecular surface, suggesting slight rearrangement of bound solvent due to slightly differing conditions of crystallization.

Background

The subtilisins are a group of alkaline serine proteases found in the culture filtrates of various species of Bacilli. Subtilisin BPN' is produced commercially by the Nagase Company, Osaka, as a crude powder contaminated with autolytic degradation products, colored materials, and, depending upon the particular batch, some other unidentified esterolytic enzymes. Other names that have been applied to subtilisin BPN' are nagarse (2) and subtilopeptidase C (3). Its amino-acid sequence (4) and three-dimensional structure (5,6) have been determined. The name subtilisin Novo designates the enzyme commercially available from Novo Industries, Copenhagen. It has also been referred to as subtilisin B (7) and subtilopeptidase B (3). Information about the species of Bacillus producing either of these enzymes is not available as they are proprietary items of commercial importance, but the work of Welker & Campbell (8) makes it appear likely that both are strains of B. amyloliquefaciens. Subtilisin Carlsberg, also called subtilisin A (7) and subtilopeptidase A (2,3), is distinctly different from but related to BPN' and Novo in amino acid sequence, physicochemical properties and enzymic activity (2,7,9,10,11). Presumably it is produced by a strain of B. subtilis. It will not be discussed further in this communication.

Kinetic studies that have been carried out with subtilisin until recently have not placed particular emphasis on comparison of the BPN' and Novo enzymes. However, Hunt and Ottesen (7) found that the two enzymes were indistinguishable in the rates at which they catalyzed hydrolysis, under a single specific set of conditions, of both N-benzoyl-L-tyrosine ethyl ester and N-benzoyl-L-leucine ethyl ester, and Ottesen and Ostergaard (12) reported that the two enzymes produce the same plakalbumin from ovalbumin. Later, Glazer (13) showed that the two enzymes have the same values of K_m and V_{max} both for hydrolysis of N-benzoyl-L-arginine ethyl ester and N-acetyl-L-tyrosine ethyl ester.

Preliminary comparative amino acid composition (2) and tryptic peptide mapping (7) studies of the BPN' and Novo enzymes showed no significant differences, a result which has been corroborated by more detailed study of tryptic peptide compositions by Olaitan, DeLange and Smith (1) who concluded that the two enzymes were probably identical. Drenth and Hol reported that crystals of DFP inhibited BPN' and Novo enzyme grown from acetone-water produced indistinguishable X-ray patterns (3).

The existing evidence thus strongly suggests that subtilisins BPN' and Novo are identical, but the possibility is not excluded that there exists some subtle distinction that might be difficult to observe in peptide mapping and composition experiments, as, for example, interchange of two amino acid residues within a single tryptic peptide fragment. In the course of our current difference-electron-density studies of binding to subtilisin BPN' by various inhibitors and virtual substrates, we decided also to look at the difference map between BPN' and Novo. The technique is sensitive enough to reveal quite clearly even a single water molecule at 2.8 Å resolution (14), and therefore presumably a difference of one carbon atom between BPN' and Novo would be evident in our 2.5 Å difference map. Probably the only sort of amino acid replacement that could not be observed by the X-ray technique is an amide difference, but then a difference of this kind very likely would have evi-

denced itself in peptide mapping experiments, unless an unlikely coincidental interchange between amide and carboxylate side chains happened to occur within a single tryptic fragment.

Experimental

Subtilisin Novo was purchased from Novo Industries, Copenhagen (batch 59). The material was purified by dissolving it in 0.05 M acetate buffer, pH 5.5, and reprecipitating with ammonium sulfate. The precipitate was redissolved in buffer and desalted by passing through a G-25 sephadex column to give a final volume of about 12 ml and protein concentration of approximately 15 mg/ml. This solution was made 30% saturated in ammonium sulfate by addition of the solid salt, the pH was adjusted to 5.9 with a drop or less of 0.1 M NaOH, and 1½ ml aliquots were set aside to crystallize at 4°C after being lightly seeded from a stock suspension of phenylmethanesulfonyl-BPN' (PMS-BPN') crystals. This crystallization procedure is almost identical to that originally used to obtain crystals of PMS-BPN' and active BPN' (5).

A 21° precession photograph of the h0l zone showed that the Novo crystals were very probably isomorphous with crystals of active BPN'. Unit-cell parameters measured with a Hilger and Watts automatic diffractometer confirmed this conclusion. The space group is C2, with a = 66.7 Å, b = 54.5 Å, c = 62.9 Å and β = 91.9°.

Reflection intensities out to a minimum Bragg spacing of 2.5 Å were measured with the diffractometer by the partial peak scan method of Wyckoff et al., (15) for each unique hkl and its Friedel mate $h\bar{k}l$. A single crystal was used for all measurements. Exactly the same data collection and processing protocol was followed as for active BPN' (5).

A difference-electron-density map was calculated using as coefficients $(F_{Novo}-F_{BPN'})$ with the centroid phases previously obtained by multiple isomorphous replacement for PMS-BPN' (5). The grid was 1 Å x 1.4 Å x 1 Å, sectioned from y = 18/40 to 53/40.

Results and Conclusions

The Novo-BPN' difference map contained no features of sufficient density to represent substitution of one amino acid for another (excepting the possibility of an amide difference), or rearrangement of any part of the structure. It is, of course, conceivable that in fact changes may actually have occurred in some poorly defined region of the electron-density map, as, for example, in a structurally disordered flexible side chain, but such a situation seems unlikely in view of the very low level of even the largest peaks and holes in the difference map. Taking this result together with the peptide mapping comparison of Olaitan, DeLange and Smith (1), it appears safe to conclude that subtilisins BPN' and Novo are identical.

In examining such a difference electron density map one must of course have some way of deciding upon a minimum level to be considered significant. Nobbs, Watson and Kendrew (14) in their 2.8 $\mathring{A}$ study of the difference between deoxy- and ferri-myoglobin chose to consider as significant any feature exceeding in absolute height three times the r.m.s. difference-density taken over all points in the unit cell. In their case, this 3σ value was 0.18 $e/\mathring{A}^3$ and they found nine such peaks or holes. A hole of -0.68 $e/\mathring{A}^3$ was interpreted as being due to loss of a sulfate ion, and another hole of -0.42 $e/\mathring{A}^3$ was interpreted as loss of a water molecule coordinated to the heme iron atom. The remaining smaller features in the map were not considered to be significant.

For our Novo-BPN' map, the 3σ value is 0.06 $e/\mathring{A}^3$, or only one-third of the corresponding value obtained by Nobbs, Watson and Kendrew (14). As would be expected for a normal distribution of a random variable, there are hundreds of points with greater than this absolute density, but none exceed 0.10 $e/\mathring{A}^3$ and none are in locations that could correspond to sterically reasonable structural differences between the Novo and BPN' enzymes. Before drawing any final conclusions, however, it must be shown that the two difference maps, myoglobin and subtilisin, are on approximately the same electron-density scale. This can be done by comparing electron-density levels on other subtilisin difference

maps that have been calculated in exactly the same way as the Novo-BPN' map but which do contain interpretable structural features. One case is the 2.5 Å difference-electron-density map obtained earlier for PMS-BPN' vs. active BPN' (5). Here, the 3σ value was 0.11 e/Å^3 and a peak of +0.63 e/Å^3 was interpreted as the added sulfonyl group, in comparison with a hole of -0.68 e/Å^3 representing a displaced sulfate ion in myoglobin. In another as yet unpublished result obtained in this laboratory, a 2.5 Å difference-electron-density map between the Z-ala-phe chloroketone derivative of subtilisin BPN' and active BPN' contains electron-density levels which are similarly consistent with the myglobin results; the 3σ value is 0.12 e/Å^3, and the highest peak is +0.41 e/Å^3 at the site of the methylene carbon of the benzyloxycarbonyl group, in comparison with a hole of -0.42 e/Å^3 at the site of a displaced water molecule in myoglobin. Several other subtilisin difference maps have also yielded comparable results. Thus, both by comparison with other subtilisin difference maps obtained in this laboratory and by comparison with numerical values of electron-density differences obtained by others for myoglobin, we can conclude that the difference map between subtilisins Novo and BPN' is essentially featureless.

We shall turn now, finally, to discuss possible interpretations of those peaks and holes that do appear on the map above the 3σ significance level of 0.06 e/Å^3, even though we have just shown that they are probably too small to represent structural changes in the enzyme molecule. The reason these marginal features are interesting is simply that they are in fact not distributed at random throughout the unit cell. The largest peak has a height of +0.1 e/Å^3 and occurs about 1 Å from the principal heavy-atom site in the $PtCl_4^=$ derivative (5), just off the end of the side chain of Met 50. Presumably it represents a disturbance due to residual errors in phase determination and is without structural significance. Nobbs, Watson and Kendrew (14) observed similar effects of height +0.32 and +0.25 e/Å^3 at the sites of gold and mercury atom substitutions. The next most prominent feature is a hole of -0.08 e/Å^3 in an

empty crevice on the surface of the molecule, between the carbonyl oxygen of Val 149 and a hitherto unassigned peak now thought to represent a water molecule on the original PMS-BPN' electron-density map. It too is probably without structural significance. Finally, we come to the large number of peaks and holes in the range ±0.06 to 0.08 e/$\mathring{A}^3$. The interesting fact is that with the exception of a single point of +0.07 e/$\mathring{A}^3$ lying in a loosely packed interior region of the molecule near Cδ of Ile 111 all the remaining features fall in two distinct groups. One comprises a string of negative density along the axis of helix G in the vicinity of Val 246 to Thr 254 which might represent a slight loosening of this helix in the Novo crystals as compared with the BPN' crystals. The second group of peaks and holes, by far the greatest number, forms a shell surrounding the surface of the molecule, and may represent a slight redistribution of solvent and salt. If they are real, both of these features could arise as a result of slightly differing conditions of salt concentration, pH and temperature in the two crystals. It is important to bear in mind, however, that these features are all smaller than approximately one-quarter of a water molecule in electron density.

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