Binding of Lanthanide Ions to Thermolysin†

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ABSTRACT: The binding of a series of lanthanide ions to thermolysin has been analyzed crystallographically by difference-Fourier methods and by refinement of the heavy atom parameters. Europium binding was studied in detail by the calculation of a three-dimensional difference electron density map at a nominal resolution of 2.3 Å. The ten rare earth ions tested readily replace calcium at each of three binding sites and displace calcium from a fourth site at which it is bound in the native enzyme. The sites occupied by the europium ions differ by 0.3–0.7 Å from those of the calcium ions which they replace. These shifts are presumed to indicate an increase in coordination number from six or seven for the calcium ions to eight or nine for europium. The sites of lanthanide binding for the series of ions tested were found to be the same within ±0.1 Å at two sites, but to change progressively within a range of about 0.35 Å at the third site. In general the substitution of Eu^{3+} for Ca^{2+} causes very little perturbation of the protein structure, the changes which do occur being localized at the metal binding sites, and involving shifts of a few tenths of an ångström. The rare earths of higher atomic number were found to cause less perturbation of the protein structure than those with lower atomic number, possibly because their ionic radii are less than or equal to that of calcium, and their preferred coordination numbers closer to those of the substituted ions.

There has recently been interest in the use of the spectral and magnetic properties of the lanthanide ions as probes of biological structure and function, particularly for those macromolecules which bind calcium. For example, Darnall and Birnbaum (1970) have shown that neodymium(III) mimics the role of calcium in the activation of trypsin, and Smolka *et al.* (1971) found that a series of the trivalent rare earth ions could be substituted for calcium in *Bacillus subtilis* α-amylase, and that the substituted enzyme was biologically active (see also Levitzki and Reuben, 1973; Darnall and Birnbaum, 1973). Also the potential of the lanthanide cations as nuclear magnetic resonance probes of biological systems has been emphasized by Morallee *et al.* (1970).

In a preliminary communication (Colman *et al.*, 1972b) we reported that the rare earths could be substituted for calcium in thermolysin, a heat stable proteolytic enzyme whose structure has recently been determined (Titani *et al.*, 1972; Matthews *et al.*, 1972a,b; Colman *et al.*, 1972a). In the earlier communication we pointed out that the replacement of calcium by the lanthanide metals might provide a useful isomorphous replacement for protein crystallography. In this report we compare, using crystallographic parameters, the relative perturbation of the thermolysin structure concomitant on the binding of ten of the lanthanide ions. In addition, the binding of Eu^{3+} to thermolysin has been studied in detail by the calculation of a three-dimensional difference electron density map at a nominal resolution of 2.3 Å.

Experimental Section

Crystals of thermolysin suitable for X-ray diffraction studies were grown as described previously (Matthews *et al.*, 1972a; Colman *et al.*, 1972a) and equilibrated with a solution of 0.01 M Tris-acetate, 0.01 M calcium acetate, and 5% v/v dimethyl sulfoxide (pH 7.3). Rare earth substitution was achieved by transferring the crystals into solutions in which the calcium acetate was replaced by 0.01 M lanthanide chloride (Alfa Inorganics) (pH 5.5). The solutions were changed every 2 days, and X-ray photographs usually taken after 4–6 days, although from some shorter term experiments essentially complete lanthanide substitution was judged to have been achieved within 2 days. Diffraction data were measured photographically (Matthews *et al.*, 1972c; Colman *et al.*, 1972a).

The rare earths used for these experiments were initially chosen to sample the lanthanide series at intervals of about three in atomic number. From this sample it appeared that the behavior at dysprosium might be anomalous, so additional lanthanides close to this element were included to bring the number of lanthanides tested to ten.

Results

(0*kl*) diffraction data to a resolution of 2.4 Å were recorded for a series of ten rare earths, and lanthanide substitution was first visualized by calculation of difference Fourier syntheses. A typical result, showing the difference between samarium–thermolysin and native thermolysin, is shown in Figure 1a. In the thermolysin space group, $P6_122$, this projection shows the projected electron density for three molecules, so each feature appears three times. In addition the three coordinates (x, y, z) of each lanthanide ion can be obtained from a single projection. Maps for thermolysin substituted with the ten rare earth ions tested are very similar. The maps show in a very clear-cut manner that replacement of calcium by europium has taken place at calcium sites 1, 3, and 4, but that at site 2 the calcium has been displaced without substitution by the rare earth (Colman *et al.*, 1972b). Calcium sites 1 and 2 are only 3.8 Å apart, and constitute a double site partially buried between two "lobes" of the molecule (Matthews *et al.*, 1972a; Colman *et al.*, 1972a). Calcium sites 3 and 4 are single binding sites and are more exposed.

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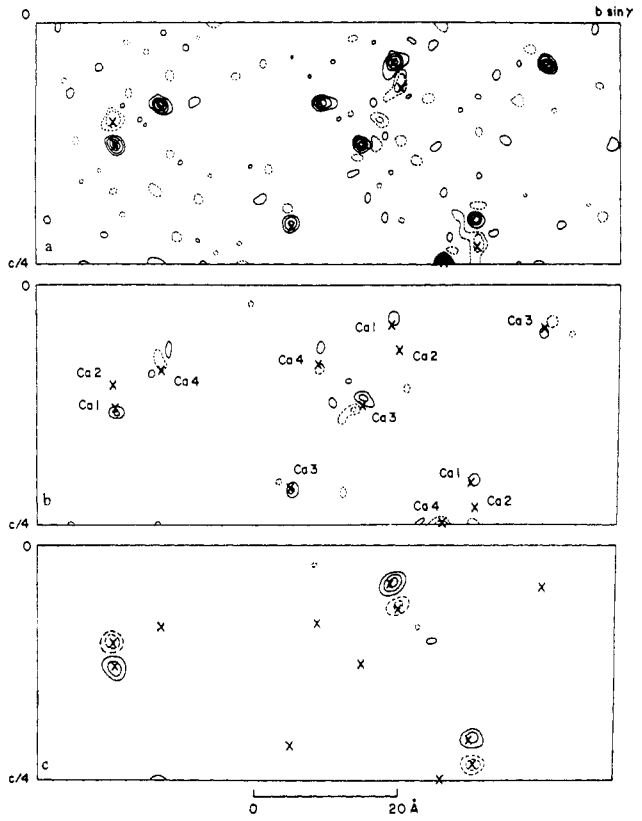

FIGURE 1: (a) Projected difference in electron density between samarium–thermolysin and native thermolysin; resolution 2.4 Å. The crosses indicate the positions of the calcium ions in the native enzyme; each site appears three times. Positive contours drawn solid, negative contours broken. (b) Projected difference density between lutetium–thermolysin and lanthanum–thermolysin. The resolution and contour level are the same as in Figure 1a. (c) Projected difference density for thermolysin crystals soaked in equimolar Ca^{2+} plus Eu^{3+}; resolution 4 Å.

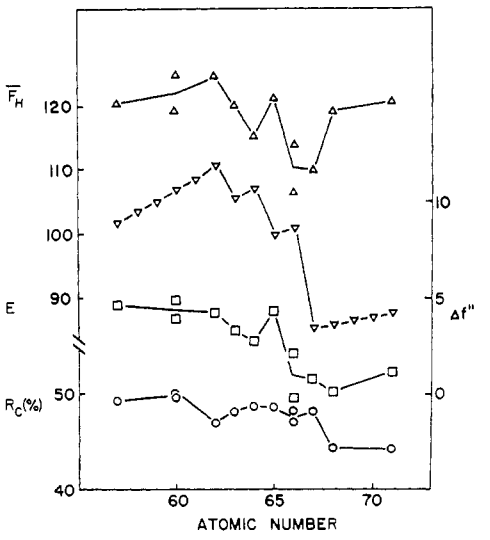

FIGURE 2: Refinement statistics for lanthanide-substituted thermolysins: (○) reliability index R_c; (□) lack of closure error E; (△) mean heavy atom scattering $\bar{F}_H$ (Table II gives root-mean-square F_H); (▽) anomalous scattering in electrons, $\Delta f''$. E and $\bar{F}_H$ are approximately in electrons.

Possible differences between the binding of the different lanthanides were further quantitated by refining the occupancy, coordinates, and "thermal parameters" of the different sites. Refinement was based on Hart's (1961) procedure, using the centrosymmetric (0kl) data. The results are summarized in Tables I and II and in Figure 2. An estimate of the standard deviations of the various refined parameters listed in Table I can be obtained from their observed variation, particularly for site 2, where the calcium ion is displaced without substitution by the rare earth ion. In this case the occupancy is negative, corresponding to a decrease in electron density, and one would expect that the refined coordinates, being those of the calcium ion before displacement, ought to be the same for all of the lanthanides. Assuming this to be the case, the observed variation in the refined parameters for site 2 leads to an estimated coordinate standard deviation of about 0.1 Å. An even more conservative estimate can be obtained by considering the variations observed for all four sites. In this case the standard deviations for x, y, and z were found to be 0.0009, 0.0009, and 0.0007, *i.e.*, 0.09 Å in each case. The standard deviations for the occupancy Z and the thermal or disorder parameter B, estimated by the same method, are 2.4 e and 4.9 $Å^{-2}$. Since the variations in the refined parameters may also reflect real changes in the lanthanide binding, the actual standard deviations may be less than those quoted above, but they should not be more. Another estimate of the reproducibility of the parameters can be obtained by inspection of the results for neodymium and dysprosium, where the lanthanide substitution, data collection, and refinement were repeated for different crystals.

The binding of europium(III) to thermolysin was analyzed in detail by calculating a three-dimensional difference map at a nominal resolution of 2.3 Å. Data were measured from a set of 22 precession photographs similar to those used for other heavy-atom derivatives of thermolysin (Colman *et al.*, 1972a). Details of the intensity statistics will be given elsewhere in connection with the use of these data in obtaining an improved electron density map of the native enzyme (B. W. Matthews, L. H. Weaver, and W. Kester, manuscript in preparation); however, it may be mentioned that the average film-to-film agreement between equivalent amplitudes on different films was 2.3%, excluding the weak reflections.

The three-dimensional difference map had as amplitudes the difference between europium–thermolysin and native thermolysin, and phase angles derived from the three isomorphous derivatives used to determine the thermolysin electron density map (Matthews *et al.*, 1972b; Colman *et al.*, 1972a). The difference electron density in the vicinity of the calcium binding sites is illustrated in Figures 3a and 3b, except in these regions the difference electron density map was featureless. On an arbitrary scale, the difference electron densities at calcium sites 1–4 were 252, −99, 201, and 204 units, respectively, while the highest other features on the map, away from the calcium binding sites, were about ±25 units, and the root-mean-square (rms) density was 7.2 units.

Table I also includes the parameters of the europium ions determined from refinement based on the three centrosymmetric zones (0kl), (hhl), and (hk0), *i.e.*, about 1500 reflections, ignoring the weak intensities which were omitted from the refinement. Refinements based on the (0kl) data alone included about 800 reflections. Occupancies are on the same scale throughout which is approximately absolute. The coordinates for the calcium sites listed in Table I were obtained from the 2.3-Å resolution electron density map of the native protein and are the coordinates of the density maxima at the respective calcium binding sites.

TABLE I: Refined Parameters for Lanthanide Ions Bound to Thermolysin.

Parameter[a]	Ca^{2+}	Eu^{3+}	La^{3+}	Nd^{3+}	Nd^{3+}	Sm^{3+}	Eu^{3+}	Gd^{3+}	Tb^{3+}	Dy^{3+}	Dy^{3+}	Ho^{3+}	Er^{3+}	Lu^{3+}
A	20	63	57	60	60	62	63	64	65	66	66	67	68	71
Site 1														
Z		30.3	31.1	31.2	33.3	30.9	31.2	30.4	33.0	31.1	30.5	31.1	36.6	39.9
B		18.0	16.0	14.1	17.1	13.1	16.3	17.3	13.2	17.7	14.2	19.3	15.1	14.5
x	750	7533	756	755	755	754	756	755	754	755	754	755	755	756
y	612	6156	618	618	618	616	618	617	616	617	617	616	617	618
z	038	0385	039	039	038	038	038	038	038	038	037	038	038	038
Site 2														
Z		−22.7	−24.9	−25.2	−23.3	−26.4	−24.8	−25.2	−25.5	−25.4	−25.4	−23.7	−24.9	−24.3
B		40.7	32.4	36.4	29.1	42.6	37.5	37.6	34.8	37.3	39.7	22.4	35.1	37.6
x	757	7580	759	760	760	760	759	760	761	760	759	759	759	759
y	626	6259	624	625	623	626	623	624	626	624	623	625	623	625
z	065	0666	067	066	066	067	066	066	066	066	066	066	066	066
Site 3														
Z		36.3	33.8	34.5	36.6	38.0	35.9	37.4	39.0	38.1	33.6	37.4	34.8	35.7
B		29.9	21.4	20.7	22.2	27.1	24.3	29.1	40.0	34.1	37.6	40.6	28.4	24.3
x	439	4325	433	433	433	433	433	432	433	433	434	435	434	436
y	875	8765	876	876	876	876	875	875	876	874	875	876	875	876
z	043	0420	041	041	041	042	042	042	042	043	043	043	043	043
Site 4														
Z		33.8	32.4	30.0	31.8	34.1	33.8	32.6	33.8	31.8	29.7	30.9	33.2	26.0
B		23.2	24.9	19.3	21.3	23.8	26.8	26.9	25.3	26.5	26.1	28.5	31.0	29.4
x	700	6999	700	700	700	700	700	700	702	701	699	698	699	699
y	486	4903	491	491	492	491	492	491	492	492	492	491	492	492
z	080	0821	084	084	084	083	083	083	082	082	082	082	082	082

[a] In the table, *A* is the atomic number; *Z* is the occupancy, approximately in electrons; *B* is the thermal parameter in $Å^{-2}$; *x*, *y*, and *z* are the fractional coordinates $\times 10^3$. The parameters in column 2 ($x, y, z \times 10^4$) are derived from refinement based on the three centrosymmetric zones (see text).

TABLE II: Refinement Statistics for Lanthanide Binding.

Parameter[a]	Ca	La	Nd	Nd	Sm	Eu	Gd	Tb	Dy	Dy	Ho	Er	Lu
A	20	57	60	60	62	63	64	65	66	66	67	68	71
f(Cu Kα)	19.7	55.0	56.3	56.3	56.0	53.1	54.8	55.7	56.2	56.2	53.1	58.7	64.1
$f_{Ln} - f_{Ca}$		35.3	36.6	36.6	36.3	33.4	35.1	36.0	36.5	36.5	33.4	39.0	44.4
$\Delta f''$(Cu Kα)	1.4	8.9	10.7	10.7	11.9	10.2	10.7	8.3	8.7	8.7	3.5	3.7	4.3
$\langle F_H \rangle$		145	144	150	150	144	139	146	128	137	132	143	145
E		89.1	86.7	89.6	87.9	84.9	83.1	87.9	73.4	81.9	77.3	75.3	78.5
R_c (%)		49.3	49.6	49.8	47.1	48.2	48.8	48.6	47.1	48.2	48.1	44.4	44.3
r_i (Å)	0.99	1.15	1.08	1.08	1.04	1.03	1.02	1.00	0.99	0.99	0.97	0.96	0.93

[a] In the table *A* is the atomic number; *f* the net real scattering, ignoring ionization; $f_{Ln} - f_{Ca}$ the difference between lanthanide and calcium scattering; and $\Delta f''$ the anomalous scattering (Cromer, 1965). $\langle F_H \rangle$ and *E* are the root-mean-square heavy-atom scattering and the lack of closure error on an approximately absolute scale. R_c is the crystallographic residual for centrosymmetric reflections and r_i is the ionic radius.

Discussion

The most clear-cut result from the studies in projection is that all the lanthanides tested readily replace calcium at three calcium binding sites, and that the sites and occupancy of lanthanide binding remain essentially constant across the spectrum of rare earths tested. As is apparent from Table I, the spread in refined coordinates is never more than 0.004, *i.e.*, about 0.4 Å, and in most cases is only half this amount.

The geometry of the respective calcium binding sites is illustrated in Figures 4a–4c. The details of the calcium coordination will be discussed in more detail elsewhere, but as is apparent from the figures, the binding sites are not obviously similar. Nevertheless, all the lanthanides tested readily replace calcium at sites, 1, 3, and 4.

To highlight any changes which might occur within the, series of rare earth ions, we calculated the projected difference in electron density between lutetium- and lanthanum-substituted thermolysin (Figure 1b). Much of the density in Figure 1a is absent in Figure 1b, confirming that the binding of the first and last lanthanides is similar, although there are features which do indicate some differences. At calcium site 1 there is positive density, presumably reflecting the increased scattering power of Lu^{3+} compared with La^{3+} (Table II).

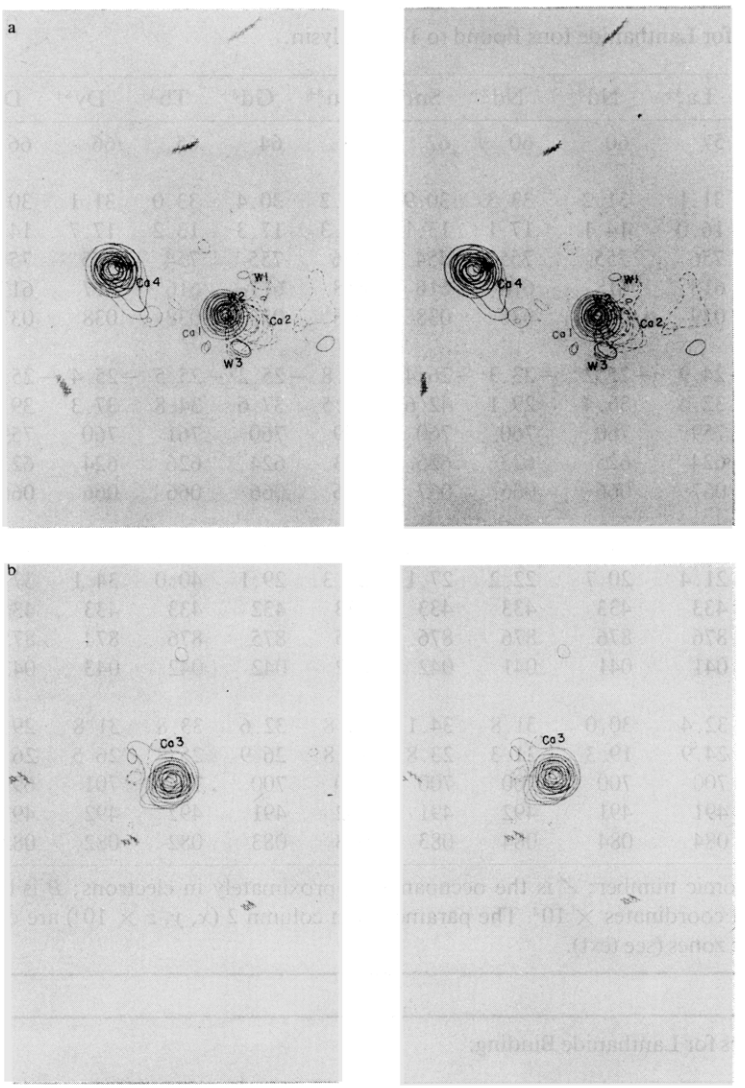

FIGURE 3: Stereopairs showing in three dimensions the difference in electron density between europium-substituted thermolysin and native thermolysin in the vicinity of the calcium binding sites; nominal resolution 2.3 Å. The contour interval is 4σ and the rows of reference marks are 9.4 Å ($a/10$) apart: (a) sites 1, 2, and 4; (b) site 3.

At calcium site 4 the density is negative, corresponding to apparent incomplete occupancy of this site by Lu^{3+}, also indicated in Table I.

It should be noted that a comparison of the degree of substitution for the different lanthanides is complicated by the fact that not only does their atomic number change, but in addition allowance has to be made for a series of absorption edges for Cu Kα X-rays which cause the apparent "real" scattering to change discontinuously across the series. Also, one should allow for the fact that a calcium ion is being displaced, and bear in mind that the occupancy tends to be correlated with the thermal parameter *B*. To facilitate comparison we have included in Table II the atomic numbers of the lanthanides, their anomalous scattering (Cromer, 1965), and their effective real scattering factor. In spite of the various complications it seems that essentially complete lanthanide substitution has occurred throughout, with the possible exception that Lu^{3+} does not fully occupy site 4.

The site of lanthanide binding at site 1 is close to that occupied by calcium, but the observed difference in coordinates (Table I), corresponding to a shift of 0.33 Å, is significant. The direction of this shift is between O^{ϵ} of Glu-177 and the water molecule ligand (Figure 4a), such that the ligand distances from O^{ϵ} of Glu-190 and the carbonyl oxygen of Glu-187 are increased. The latter two ligand distances are in fact the shortest for Ca-1.

It seems reasonable to assume that the shift between the site of calcium and lanthanide binding is indicative of a change in coordination number. In thermolysin, Ca-1 appears to have distorted octahedral (sixfold) coordination, whereas with oxygen ligands coordination numbers of 8 or 9 are expected (Pauling, 1960) and usually observed for the rare earth ions (*e.g.*, see Helmholz, 1939; Hoard *et al.*, 1965; Kay *et al.*, 1972; Martin and Jacobson, 1972a,b; Moeller *et al.*, 1968, and references therein). There is no indication in the difference electron density map of europium minus native thermolysin (Figure 3a) that when europium replaces Ca-1 water molecules serve as additional ligands at this site. Also there is no indication of a substantial rearrangement in the positions of the amino acid side chains of Asp-185, Glu-177, or Glu-190 associated with the substitution of europium for calcium. Nevertheless, inspection of the geometry of these side chains (Figure 4a) suggests that with very little movement, one or more of them could act as bidentate ligands, increasing the coordination number of the lanthanide ion at site 1. This hypothesis would also explain the inability of a second lanthanide ion to bind at site 2, for if one or more of the acid groups listed above coordinated exclusively the lanthanide at site 1,

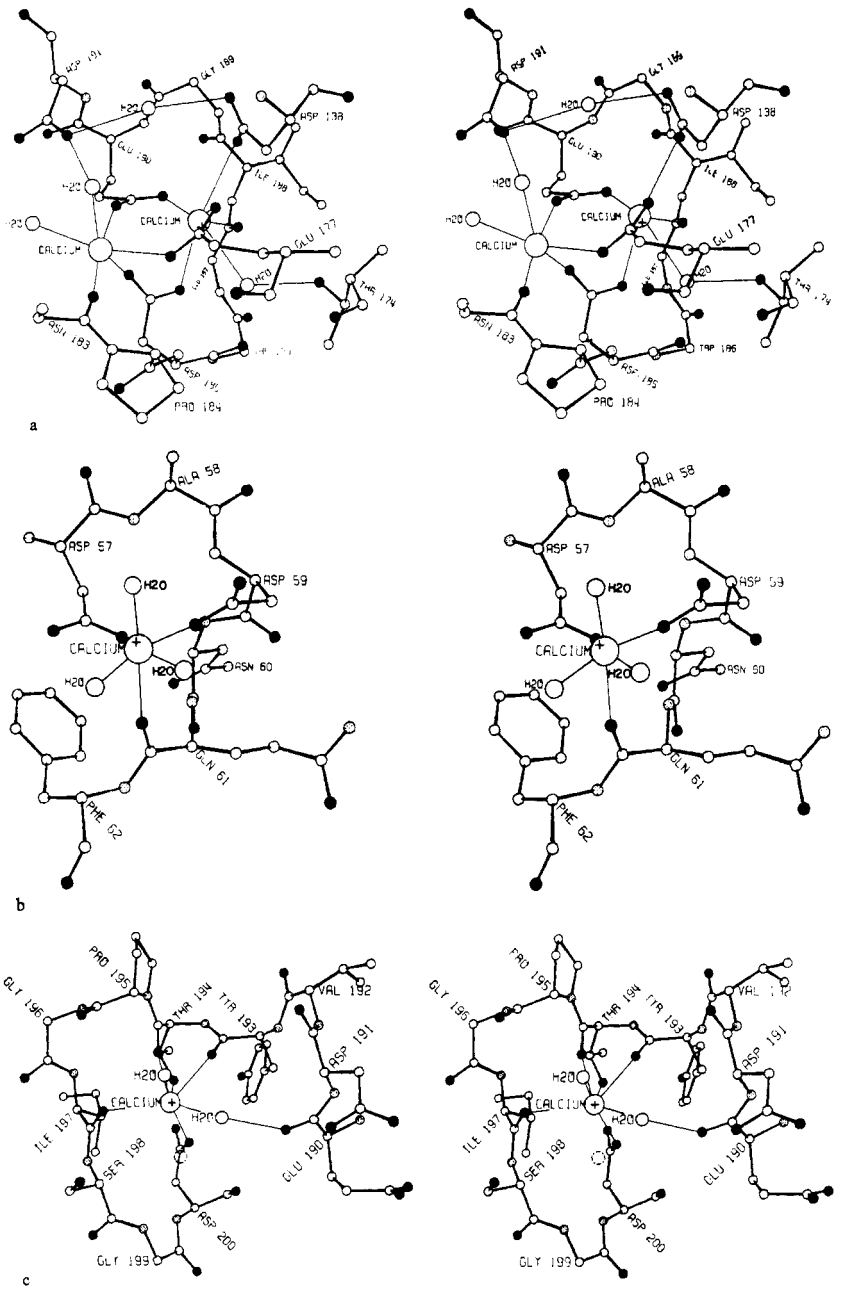

FIGURE 4: Stereodrawings illustrating the geometry of the calcium binding sites. Carbon atoms are represented by open circles, oxygen by solid circles, and nitrogen atoms are drawn stippled. Water molecules and calcium ions, indicated by larger circles, are labeled. The calcium ligands are indicated by thin lines, and the positions occupied by bound europium ions are shown by crosses: (a) sites 1 and 2; (b) site 3; (c) site 4. An additional water molecule bound when europium substitutes for calcium (see text) is shown as a broken circle.

then the potential protein ligands available at site 2 would be reduced from four as in the binding of Ca-2 to perhaps one or two. It might be noted that there is a precedent for "double" lanthanide binding in complexes of lanthanide ions with the nicotinate and isonicotinate ions (Moore *et al.*, 1972; Kay *et al.*, 1972). In these complexes the rare earth ions approach within about 4.2–4.5 Å and are bridged by four nicotinic acid groups. In the crystal structures longer Ln–Ln approaches also occur, but in these cases water molecules participate in the coordination. The distance of 4.2 Å is not that much greater than the Ca-1–Ca-2 distance of 3.8 Å observed in thermolysin, but, in contrast, the calcium ions in thermolysin are bridged by three rather than four acid groups, *i.e.*, those of Asp-185, Glu-177, and Glu-190 (Figure 4a). As pointed out by Kay *et al.* (1972), a carboxylic acid group occupies a smaller volume than two oxygens from different ligands, and it could well be that once a lanthanide ion is complexed at site 1, the number and density of potential ligands available at site 2 will not support a second ion at this position. It is possible that one or more of the oxygen atoms could coordinate two calcium or lanthanide ions, as, for example, in the crystal structure of $CaCl_2 \cdot$glycylglycylglycine (Van der Helm and Willoughby, 1969), but the principle of stronger coordination at site 1 being at the expense of coordination potential at site 2 would still apply.

Presumably ionic interactions also tend to prevent a second lanthanide ion binding at site 2, for once the first trivalent ion is bound, the residual negative charge in the region of the double site is less than for calcium. Also the ionic repulsion between two lanthanide ions, or between a lanthanide and calcium ion, is greater than that for a pair of calcium ions.

One trend in the refined lanthanide coordinates (Table I)

which might be significant occurs at calcium site 3 where, in going from lanthanum to lutetium, there is an apparent progressive shift of about 0.35 Å, made up of 0.3 Å along x and 0.25 Å along z. This apparent shift is also indicated by the positive and negative peaks which straddle this site in the electron density map (Figure 1b) showing the projected difference in density between lutetium- and lanthanum-substituted thermolysin. The direction of the shift is such that the larger lanthanide ions occupy positions successively further from that of calcium in the native enzyme. The distance from Ca-3 to the lutetium site is about 0.35 Å, increasing to about 0.7 Å for europium and the larger ions. As shown in Figure 4b, the shift is approximately along the ligand direction from the side chain of Asp-59 to the calcium site. In the three-dimensional difference map there are weak, but probably significant positive and negative density features adjacent to the calcium site 3 (Figure 3b) which are consistent with a slight movement of the side chain and backbone of Asp-59 toward calcium site 3, concomitant on the substitution of Eu^{3+} for Ca^{2+}. It appears that there is a small rearrangement of the protein structure in the vicinity of Asp-59 such that the distance from the side chain of this residue to the bound ion tends to remain approximately constant. The shift in the ion binding site, and presumably also in Asp-59, increases for the lanthanides of larger radii, consistent with the decrease in isomorphism observed for the larger ions. The site occupied by Eu^{3+}, for instance, is such that Asp-57 may act as a bidentate ligand, rather than monodentate as for calcium. Also, although the exact position occupied by Asp-59 in the Eu^{3+}-substituted enzyme is not certain, it may well be that the movement of this residue described above results in bidentate ligancy. Thus the coordination number of Eu^{3+} at site 3 could, as at site 1, be at least as high as eight.

At site 4 there is also a small but significant difference in the site of calcium and lanthanide binding, in this case amounting to 0.5 Å. The direction of this shift, as seen in Figure 4c, is roughly toward the viewer, *i.e.*, such that when the lanthanide ion is bound, the protein ligand distances are slightly longer than for calcium. The small "tail" of density seen at calcium site 4 in the three-dimensional map (Figure 3a) extends in a direction toward the peptide nitrogen of Gly-199 (Figure 4c) and is probably due to a water molecule coordinated by the europium ion, but apparently not by calcium. As a consequence the coordination number of Eu^{3+} at this site would be eight, compared with seven for Ca-3.

In the difference map in the vicinity of the double calcium binding site there are two weak negative peaks, labeled W1 and W2, and a positive peak W3. The negative features indicate the loss or partial loss of the two water molecules which in the native enzyme act as ligands for Ca-2 (Figure 4a). Feature W3 in Figure 3a is thought to be due to a water molecule which is bound by the europium-substituted enzyme. In the native enzyme W1 is hydrogen bonded to the carbonyl oxygen of the peptide between Lys-182 and Asn-183. This peptide group lies midway between the positive and negative density seen to the right of Ca-2 in Figure 3a, indicating that when the water molecule is displaced, the polypeptide backbone moves slightly away from the calcium binding site. This adjustment, and those in the vicinity of sites 1 and 3, discussed above, appear to involve shifts of at most a few tenths of an ångström. With these exceptions the remainder of the protein appears to be entirely unaffected. As is expected, the refined coordinates for Ca-2, the ion displaced when lanthanides are bound, agree closely with the coordinates of this ion in the native enzyme (Table I), providing a useful check on the accuracy of both sets of parameters.

In Table II and in Figure 2 we have shown several crystallographic indices which may be used to measure the departure from isomorphism of the native and the lanthanide-substituted enzyme, concomitant on binding the various lanthanides. From the fact that R_c, the crystallographic residual for centrosymmetric reflections (Cullis *et al.*, 1961), is in each case less than 50%, it is clear that by the usual crystallographic standards the isomorphism is quite good, and that any of the lanthanides tested would be satisfactory as an isomorphous derivative for phase determination. To obtain maximum benefit from both isomorphous replacement and anomalous scattering measurements, the rare earth of choice would be samarium ($\Delta f'' = 11.9$ e), although several other lanthanides have anomalous scattering components in excess of ten electrons, and would serve almost as well. Recently Sieker *et al.* (1972) used a samarium derivative in the structure determination of a bacterial ferredoxin, and Watenpaugh *et al.* (1972) were able to obtain an interpretable electron density map of Flavodoxin from *Desulfovibrio vulgaris* based exclusively on a single samarium derivative.

In contrast to the reliability index R_c, which is a ratio, the lack of closure error E (Blow and Crick, 1959) provides an absolute measure of the perturbation of the structure on binding the heavy atoms. It will be noted in Table II and in Figure 2 that E decreases sharply at dysprosium ($A = 66$) and has lower values for the lanthanides with atomic numbers above 66 than for those below. The sharp drop at dysprosium is not apparent in R_c, although the residual does drop at erbium ($A = 68$). The detailed interpretation of these apparent changes is complicated by the fact that they may be due in part to differences in the apparent occupancy of the lanthanide sites, reflected by the $\bar{F}_H$ values. Nevertheless, it seems clear that there is a significant decrease in the perturbation of the structure for the lanthanides of higher atomic number than those of lower atomic number.

A partial explanation of this phenomenon might be that it is due to the different size of the lanthanide ions. Going from lanthanum to lutetium, the ionic radius decreases from 1.15 to 0.93 Å, and the radius of dysprosium, the element at or near where the decrease in structural perturbation occurs, is 0.99 Å, the same as that of calcium (Pauling, 1960). However, the present study shows quite clearly that in each of three substitutions the site of lanthanide binding differs from that of calcium by about 0.5 Å, and also suggests that the coordination is modified. Therefore, an explanation based on equality of ionic radius of the substituting ion is not in itself sufficient.

Another possible contribution to the more-perfect isomorphism observed for the smaller lanthanide ions might be a change in coordination number within the lanthanide series. Moeller *et al.* (1968) have speculated that such a change is responsible for the nonmonatomic behavior of the thermodynamic properties of certain rare earth complexes across the series. However, if, as a consequence, lanthanum were to coordinate one more water molecule than lutetium when bound to the protein, then this water molecule might be seen in a map showing the difference between these two substitutions. The actual map (Figure 1b) shows no such indication. The fact that refined coordinates of the lanthanide ions (Table I) do not change abruptly at any point within the series tends to rule against a discontinuous change in coordination geometry, but a gradual adjustment of the protein ligands to achieve higher coordination cannot be ruled out.

We have also described above how the localized changes in the vicinity of site 3 appear to be greatest for the larger

lanthanides and no doubt contribute to the decrease in overall isomorphism observed for the larger ions. In summary, the reason why the smaller ions cause less perturbation of the thermolysin structure is not clear, but presumably derives from a combination of ionic radius, coordination preference, and localized stereochemistry.

The X-ray experiments do not provide binding constants for the calcium or europium binding sites; however, an attempt was made to compare the relative affinity of calcium and europium by soaking crystals of thermolysin for several days in a solution 0.01 M in both ions. The resultant 4-Å resolution difference electron density map (Figure 1c) shows that little if any europium substitution has taken place at sites 3 or 4, but that at the double calcium site, even in the presence of equimolar calcium, europium substitution and calcium displacement have occurred. It can be concluded that the affinity of calcium for sites 3 and 4 is higher than that of europium, but that the affinity of europium for site 1 is higher than the combined affinity of calcium for sites 1 and 2.

There have been several reports in the literature dealing with the ability of the lanthanide ions to mimic the biological function of calcium, for example, in the activation of trypsinogen (Darnall and Birnbaum, 1970) and of α-amylase (Smolka *et al.*, 1971; Darnall and Birnbaum, 1973). The present report shows directly for the first time that, at least for thermolysin, the substitution of calcium by the lanthanides results in a minimal perturbation of the protein structure. For thermolysin, the presence of calcium is essential for thermostability (Endo, 1962), although the metal is not directly involved in catalytic activity (Feder *et al.*, 1971; Matthews *et al.*, 1972a; Colman *et al.*, 1972a). In unpublished experiments, kindly performed by Dr. J. Feder, it was found that crystals of the europium-substituted enzyme, when dissolved, had normal proteolytic activity, and were much more thermostable than "one calcium" thermolysin (Feder *et al.*, 1971), although not as stable as dissolved crystals of the normal four-calcium enzyme. Thus, at least to some degree, europium mimics the biological role of calcium in thermolysin. Smolka *et al.* (1971) reported that for *Bacillus subtilis* α-amylase, the smallest rare earth ions were most effective as calcium ion substitutes in reactivating the apoenzyme. If the X-ray results for thermolysin are an indication, it would seem that the smaller lanthanides are more effective because they perturb the structure least, not necessarily because they form tighter complexes, as suggested by Smolka *et al.* (1971).

The present study demonstrates that although lanthanide ions can replace calcium ion proteins with minimal perturbation of the structure, the detailed interpretation of the effectiveness of the lanthanide ions in replacing calcium function is complicated not only by the charge difference and size variation, but also by possible changes in the coordination number and the geometry of binding.

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