IDENTIFICATION OF THE CHEMICAL DIFFERENCE BETWEEN CHROMATOGRAPHIC COMPONENTS OF RIBONUCLEASE

by

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The chromatography of crystalline ribonuclease[1 2 3 4] invariably shows the presence of more than one component. The leading, and usually the major component, has been called ribonuclease A[3]. A second component(B) has also been shown to have enzymic activity. Several components have also been observed by zone electrophoresis[5].

In recent work with this enzyme in this laboratory, utilizing crystalline ribonuclease prepared by Armour and Co., one lot was encountered which contained the first and second chromatographic components in about equal amounts. This provided an opportunity to isolate each component separately in sufficient quantity (about 0.5 gram) so that quantitative studies could be performed to determine the chemical difference between these components.

It was found that all our results are compatible with a difference of a single carboxyl group. No evidence was found to confirm LEDOUX's suggestion[4] that oxidation of sulfhydryl groups is involved.

EXPERIMENTAL

Crystalline ribonuclease was obtained from Armour and Co. Lots 381-059 and 381-062 were used, lot 381-062 being the one from which two components were isolated. An assay performed for us by Dr. GEORGE KALNITSKY indicated that both lots had normal enzymic activity, that of lot 381-062 being slightly higher. Chromatograms were obtained on the preparative columns described by HIRS, STEIN AND MOORE[3], using Amberlite XE-97 ion exchange resin. Ultraviolet light absorption was used as a measure of protein concentration in successive portions of the eluate.

All stock solutions used, whether of the original crystalline protein or of the separated components were passed repeatedly through mixed-bed ion exchange columns of the type described by DINTZIS[6] until no further change in pH took place. The stock solutions were then regarded as entirely salt-free and also isoionic. The protein concentration in stock solutions was determined by drying aliquots to constant weight at 107° C.

Titration data were obtained by the method previously described[7]. A Beckman Model GS pH meter was used. Spectrophotometric titration of phenolic groups was by the method of CRAMMER AND NEUBERGER[8], as modified by TANFORD AND ROBERTS[9] for Beckman spectrophotometers. All experiments were at 25° C. The usual precautions were taken to exclude carbon dioxide.

RESULTS

The chromatograms obtained from solutions of the two lots of ribonuclease are shown in Fig. 1. Lot 381-059 is seen to resemble crystalline ribonuclease as examined by MARTIN AND PORTER[1] and by HIRS, STEIN AND MOORE[2 3]. It contains close to

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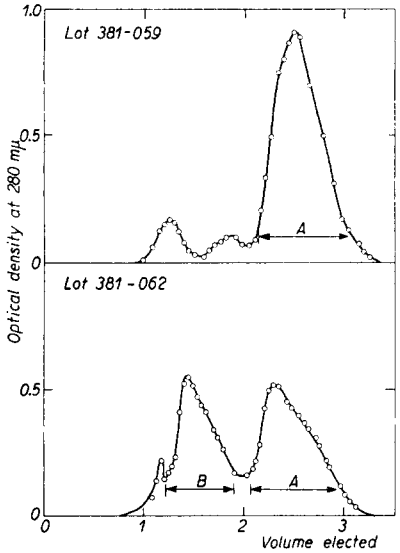

Fig. 1. Chromatograms for Lots 381-059 and 381-062 obtained from the preparative column of HIRS, STEIN AND MOORE. The arrows indicate portions of the eluate used in the study of the separated components.

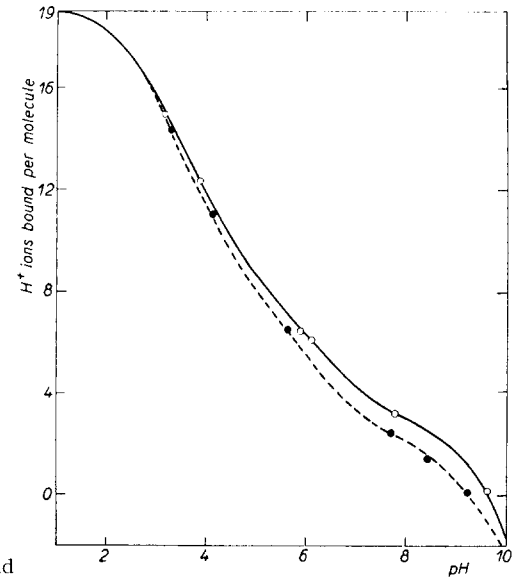

Fig. 2. The titration curve of unfractionated lot 381-059 (solid line), of component A (open circles), and of component B (solid circles).

90% of the major component, ribonuclease A. Lot 381-062, on the other hand, is seen to consist largely of two components in about equal amounts. We shall designate these as components A and B. The portions of the eluate used in our studies of the separated components are also shown in Fig. 1.

The first indication of a difference between the lots used was a marked difference in their isoionic points, measured by dissolving a portion of the isoionic stock solution in 0.15 *M* KCl. This difference was accentuated when the separated components A and B were measured, as shown by Table I. It should be mentioned that ribonuclease does not appear to combine appreciably at the isoionic point with either potassium or chloride ion, so that the effect of ionic strength on the data of Table I would be quite small[10], *i.e.* the particular ionic strength used to measure the pH is unimportant.

TABLE I

ISOIONIC POINTS MEASURED IN 0.15 *M* KCl AT 25° C

Ribonuclease, Lot 381-059	9.63
Ribonuclease, Lot 381-062	9.47
Component A from Lot 381-059	9.65
Component A from Lot 381-062	9.65
Component B from Lot 381-062	9.23

The difference in the isoionic points of components A and B means that component B has either fewer cationic groups than component A or that it has more anionic groups. To distinguish between these possibilities as many points on the titration curve of each component were obtained as the amount of available material permitted. The results, together with a curve for the unfractionated lot 381-059, are shown in Fig. 2.

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It can be seen that both components have similar titration curves. The difference of 0.42 between the isoionic points corresponds to the difference in pH required to titrate a single ionizable group, so that the difference between the two components in number of cationic or anionic groups appears to be just a single group.

Fig. 2 shows that the titration curves of both components approach the same acid end point. Since the numberof groups titrated between the isoionic point and the acid end point is a measure of the total number of cationic groups per molecule, this indicates that the difference between the components cannot be due to a difference in basic nitrogen groups. This leaves a difference in the number of carboxyl groups as the most reasonable explanation for the difference in isoionic points. That this is indeed the difference is confirmed if the experimental points of Fig. 2 are replotted as a differential titration curve, as shown in Fig. 3. This clearly shows that the single group distinguishing the two components is a group with apparent pK 4.7 $\pm$ 0.4, the probable error of 0.4 being a result of the general uncertainty of about 0.1 in the number of groups titrated at a given pH. This pK is in the range characteristic for carboxyl groups.

Fig. 2 also shows that the titration curve of component A is, within experimental error, identical with that of unfractionated ribonuclease from lot 381-059. In view of the experimental uncertainty of 0.1 to 0.2 in the number of groups titrated at any pH, this result is not surprising for the presence of about 10% of component B, differing from component A by only one carboxyl group, would create a difference of only 0.1 such group between the pure component and the unfractionated sample. It should be noted (Fig. 1) that a small amount of a third component is also present. The close correspondence between the titration curves of component A and the unfractionated sample suggests that this third component, too, cannot differ very markedly from the other two.

Our remaining experiments are designed to indicate whether or not any additional difference between the components A and B can be detected. None was found: viscosity and light-scattering studies, for example, showed essentially no difference between lots 381-059 and 381-062, indicating that the two components have the same molecular weight and hydrodynamic volume.

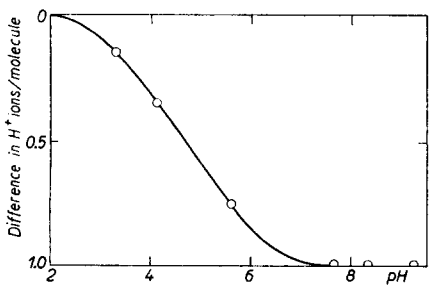

Fig. 3. Difference in titration curve between components A and B.

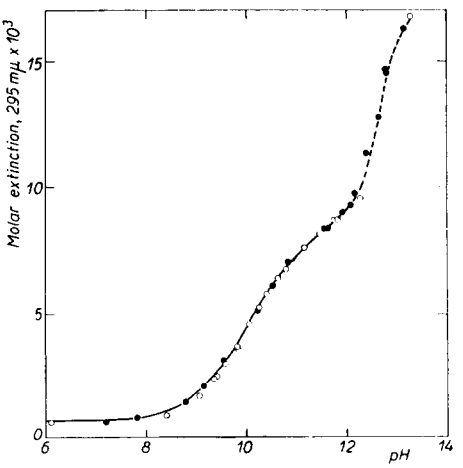

Fig. 4. Spectrophotometric titration of the phenolic groups of lot 381-059 (solid circles) and lot 381-062 (open circles).

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A particularly sensitive test is provided by a spectrophotometric study of the ionization of the phenolic groups. As will be shown in a detailed analysis to be presented at a later date, the course of this ionization in ribonuclease is very unusual in that one half of the phenolic groups appear to ionize reversibly in the range of pH 9 to 11, while the other half ionize irreversibly above pH 11. Fig. 4 shows that lots 381-059 and 381-062 show identical behavior in this regard, from which one can see not only that the number of phenolic groups per molecule must be the same in both lots (and, hence, in both components), but also that the same division of these groups into two types occurs. Furthermore, the precise location and form of the spectrophotometric titration curve is essentially the same.

Actually a small difference should exist between the two lots. Because of the charge difference in the alkaline pH region, points from lot 381-059 should lie 0.02 pH units to the *left* of corresponding points from lot 381-062. A difference of this order of magnitude is in fact observed when an enlargement of Fig. 4 is examined. The scatter of points is sufficiently great, however, so that this difference is not statistically significant.

DISCUSSION

The results presented above not only show that there is a difference of one in the number of free carboxyl groups in ribonuclease A and B, but also suggest that this is the only difference. It is possible, of course, that there is also a difference in the number of non-titratable amino acid residues. However, ribonuclease is a relatively small molecule, and it is difficult to see how appreciable changes in the number or order of amino acid residues could occur without effect on molecular weight, viscosity, and such intimate structural details as the shielding of one half of the phenolic groups from attack by acids or bases. In particular, the slope and precise location on the pH scale of a titration curve such as those shown in Figs. 2 and 4 are easily affected by minor changes, in other portions of the molecule.

It is of interest to note that a difference in the number of free carboxyl groups can arise without change in the total number of amino acid residues by substitution of a glutamic or aspartic acid side chain for one of glutamine or asparagine. One could easily visualize such a substitution arising as a result of hydrolysis during the extraction of ribonuclease from the pancreas, for the extraction procedure[11] involves both exposure to relatively strong acid (0.25 M H_2SO_4) and heating to 100° C. However, MARTIN AND PORTER[1] and HIRS, MOORE AND STEIN[3] have demonstrated that the components of ribonuclease do not arise in this way, but are more likely already present in the original pancreatic material.

In addition to ribonuclease, at least two other proteins of low molecular weight contain two components. Insulin is one of these: its components have been separated by countercurrent distribution[12] and have been shown to differ precisely in the same way as is here suggested for ribonuclease, *i.e.*, by substitution of a free acid side chain for an amide side chain. The difference between components of lysozyme has also been ascribed to this cause[13].

If the conclusions of the present paper are correct, and they should be tested by amino acid analysis of the separated components, they are testimony to the extraordinary power of the method of chromatographic separation developed by

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STEIN AND MOORE and co-workers. That so clean and easy a separation can be obtained of two macromolecules differing by so little is a remarkable achievement.

It should be noted that the conclusions of the present paper disagree with the suggestion of LEDOUX[4] that the difference between the chromatographic components of ribonuclease lies in a difference in the state of oxidation of sulfhydryl groups. Also arguing against LEDOUX's suggestion is the recent amino acid analysis by HIRS, STEIN AND MOORE[14], which showed no sulfhydryl groups to be present.

ACKNOWLEDGEMENT

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SUMMARY

Differences in the isoionic points and titration curves of two chromatographic components of crystalline ribonuclease can be ascribed quantitatively to a difference of one in the number of free carboxyl groups. Other quantitative properties of the components agree so closely that it is probable that no other difference between the components exists.

RÉSUMÉ

Les différences dans les points isoioniques et dans les courbes de titrage des deux constituants chromatographiques de la ribonucléase cristallisée peuvent être attribuées quantitativement à une différence de un dans le nombre des groupes carboxyliques libres. Les autres propriétés quantitatives des constituants sont si voisines qu'il est probable qu'il n'existe aucune autre différence entre eux.

ZUSAMMENFASSUNG

In den isoionischen Punkten, sowie in den Titrierungskurven von zwei chromatographischen Ribonukleasekomponenten festgestellte Unterschiede können quantitativ darauf zurückgeleitet werden, dass die eine Komponente eine freie Karboxylgruppe weniger als die andere enthält. Andere quantitative Eigenschaften der Komponenten stimmen so eng miteinander überein, dass wahrscheinlich keine weiteren Differenzen zwischen ihnen bestehen.

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