The Quaternary Structure of Yeast Aminopeptidase I

2. Geometric Arrangement of Subunits

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(Z. Naturforsch. **32 c**, 938–943 [1977]; received August 2, 1977)

Aminopeptidase, Yeast, Electron Microscopy, Quaternary Structure

Electron micrographs of native aminopeptidase I and of isolated subfragments were taken after negative staining with uranyl formate. From these studies and from the chemical evidence summarized in the preceding paper it is concluded that the active enzyme is a dodecamer possessing pseudo-D_3 symmetry with the dimer as the smallest symmetric unit.

Introduction

In order to establish the quaternary structure of an oligomeric protein two main questions have two be answered. In the first place the number of protomers from which the native protein is assembled has to be established. This has been done with a multitude of proteins; from the results of these studies it seems now well documented that the great majority of oligomeric proteins is made up of an even number of identical subunits [1]. However, from experiments of this kind no direct information is available on the spatial arrangement of the subunits or on the symmetry of the complex. As an elegant approach to this problem the electron microscopic examination of the particles comes into consideration, provided their molecular weights exceed 2×10^5. For the visualization of macromolecules the negative staining technique has been widely employed [2]. Although the interpretation of molecule images created in this way may be difficult, rather straightforward results have been obtained with a considerable number of multi-subunit proteins [3]. In the preceding paper [4] results on the molecular weights of aminopeptidase I from yeast and of its main subfragments have been presented. It was shown that the active enzyme is a dodecamer assembled from only one type of subunits with a molecular weight of 5.3×10^4. In addition, the inactive hexameric form and the dimer of the aminopeptidase protomer are found in neutral or moderately acidic enzyme solutions. The present report deals with electron microscopic studies on the shape and geometry of the various aminopeptidase forms. A model describing the organization of the enzyme on the quaternary structure level is proposed.

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Materials and Methods

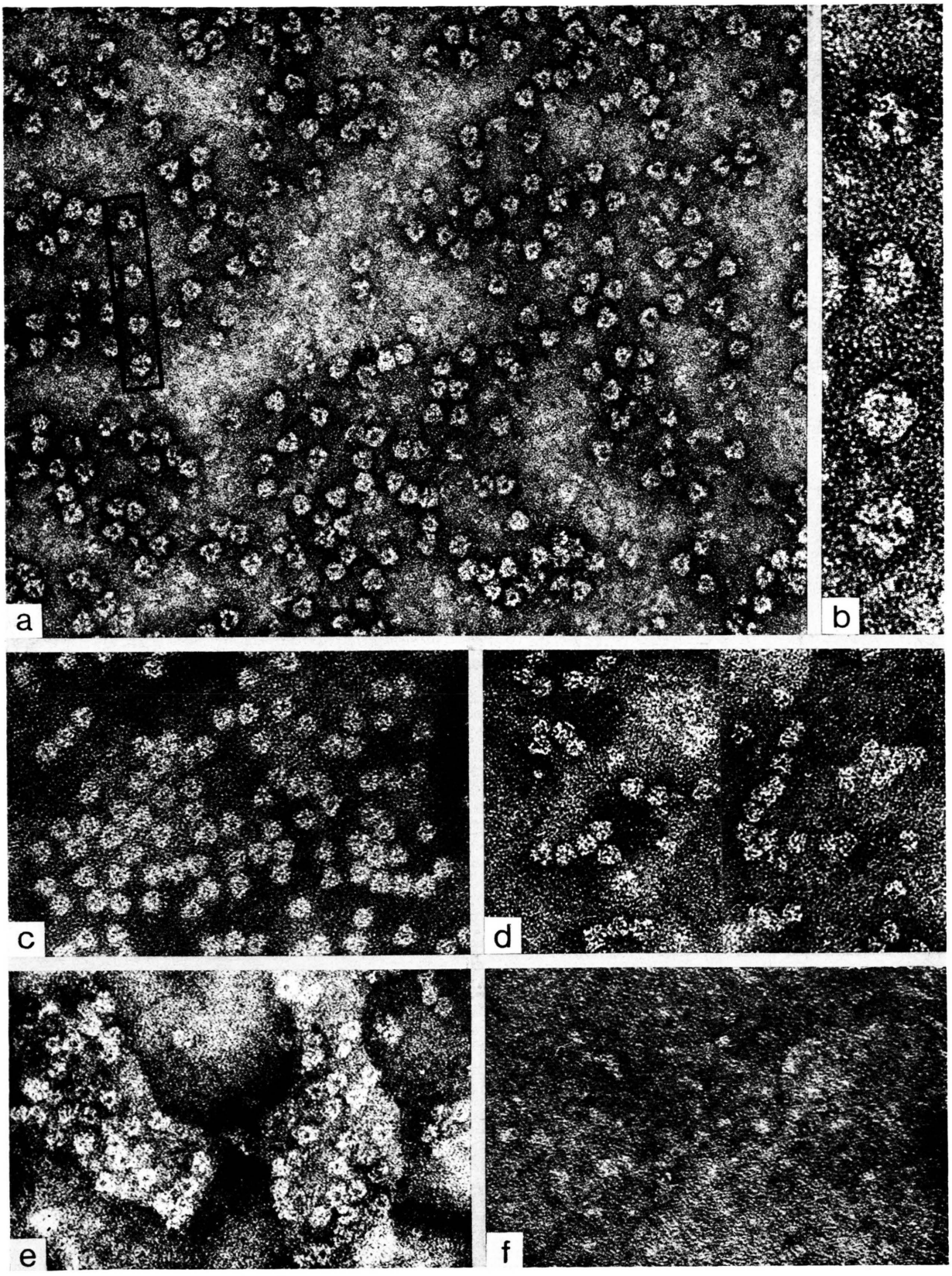
Aminopeptidase I was purified from yeast autolysates as described elsewhere [5]. Separation of the various molecular forms was carried out by sucrose density gradient centrifugation (*cf* the accompanying paper). Immediately after fractionation of the gradients the enzyme samples scheduled for electron microscopic examination were freed from sucrose and concomitantly brought to the desired protein concentration by repeated dilution with buffer (usually volatile ammonium acetate buffers were used) and concentration by ultrafiltration through Amicon PM 10 membranes. Further details on the composition of aminopeptidase samples are found in the legend to Fig. 1.

Specimens for electron microscopy were prepared as follows: Copper grids (400 mesh) coated with polyvinyl formal (Mowital F 40, Farbwerke Hoechst AG) were immersed in the respective enzyme solution for 30 sec to 2 min, then incubated for 1 min in a 2% aqueous solution of uranyl formate (Serva, Heidelberg) or 1% sodium phosphotungstate, pH 7.2, and finally air-dried. Electron micrographs were taken with a Siemens Elmiskop 101 operated with 80 kV and equipped with pointed filament and an anticontamination device. The original magnification was 100 000 in all cases.

Results

1. Electron micrographs

The appearance of negatively stained aminopeptidase forms in electron micrographs is shown in Fig. 1. Clearly, preparations contrasted with uranyl formate (Fig. 1 a – b) or phosphotungstate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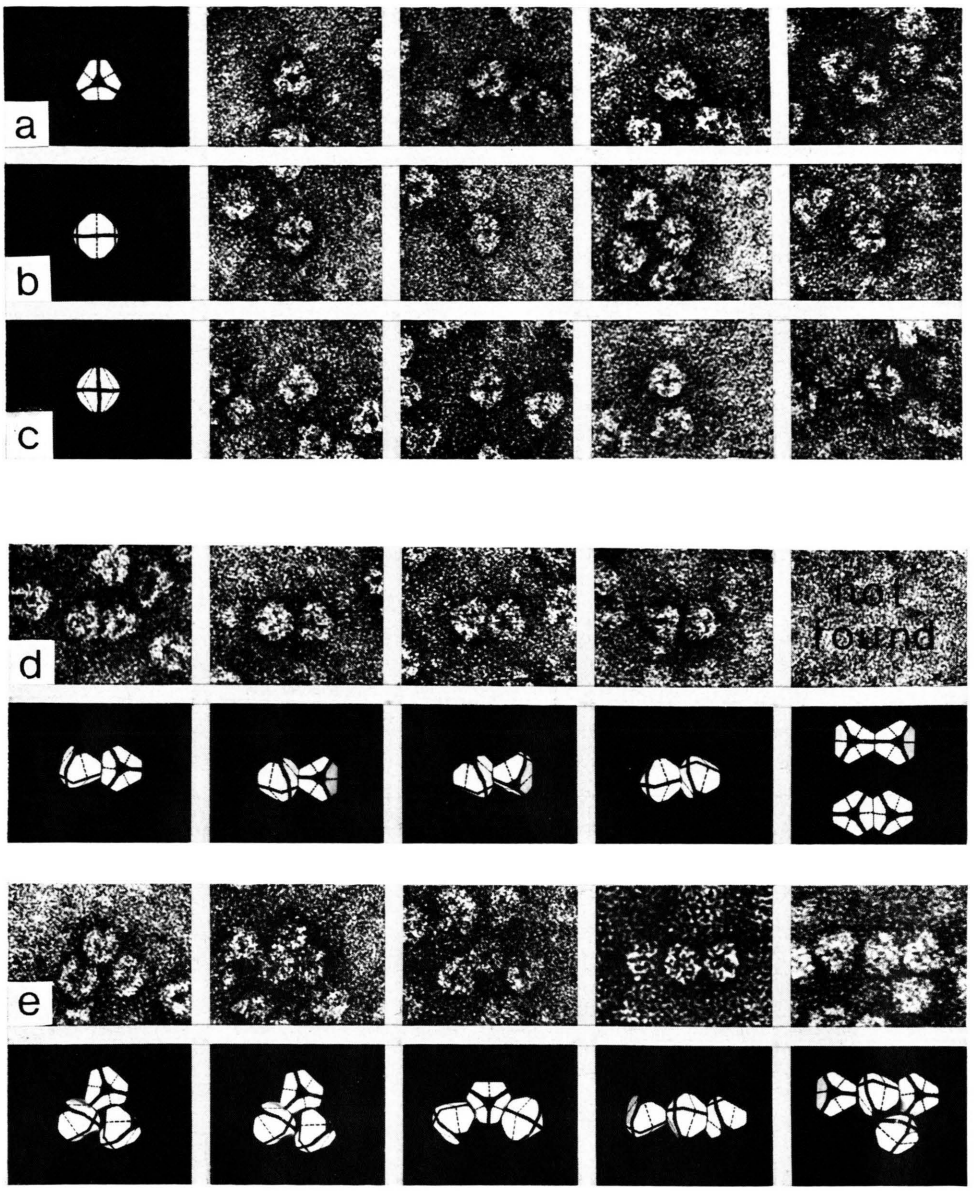

Fig. 2. Comparison of aminopeptidase particle projections with models (cf. Fig. 3). a) Particles viewed down the three-fold axis (group A). b) Particles viewed along at two-fold axis (group B). c) Particles viewed down the same two-fold axis but from the opposite direction (group C). d) – e) Aggregates, composed of two, three or four 22S-particles. Magnification: × 500 000.

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Fig. 1. Electron micrographs of yeast aminopeptidase I. The particles were negatively stained with uranyl formate (a, b, d – f) or sodium phosphotungstate (c). Magnification: × 350 000 (a, c – f), × 1 000 000 (b).

a) Native aminopeptidase (1.5 mg/ml in 50 mM ammonium acetate buffer, pH = 6.2, containing 100 mM Cl^- and 50 μM Zn^{2+}).

b) Higher magnification of the field outlined in a).

c) Same preparation as in a) but stained with phosphotungstate.

d) Same preparation as in a) reexamined after six weeks of storage at 4 °C.

e) Isolated 12.5S fragments (0.6 mg/ml in 100 mM ammonium acetate buffer, pH = 6.2).

f) Isolated 6S fragments (0.2 mg/ml in 100 mM sodium acetate buffer, pH = 4.0).

(Fig. 1 c) do not significantly differ from each other with respect to the size and general shape of the particles. The preservation of the molecules and the resolution of substructures, however, is much better with the uranyl formate stain. Therefore in most experiments the latter method was employed.

The enzyme sample from which Fig. 1 a – b and Fig. 2 were derived was obtained by pooling the active fractions of a Sepharose 6B column eluate. About 70% of the particles present in such a solution are active 22S molecules with a molecular weight of 6.4×10^5 (see the preceding paper). A spherical molecule with this molecular weight and a partial specific volume of 0.725 cm^3/g (*cf* ref. 5) will have a diameter of about 130 Å. The observed particle dimensions (100 – 110 Å mean diameter) are in good agreement with this value. Fig. 1 d shows that aminopeptidase molecules have a marked tendency to aggregate upon standing in concentrated solutions. However, in freshly prepared samples aggregates of two or three particles are also found (Fig. 1 a). A closer inspection of the images encountered in Fig. 1 shows that the projections may be classified in three main groups. (Some typical examples are compiled in Fig. 2 a – c; Fig. 1 b shows a small section of Fig. 1 a containing one projection of each group in a higher magnification.)

Group A: Images of triangular shape (*i. e.* with three-fold symmetry) made up from three equal components and showing a central hole filled up with stain. Corner-to-corner distance: 100 Å (*cf* Fig. 2 a).

Group B: Slightly elliptic particles with an apparent two-fold symmetry, intersected by a dark band of stain (*cf* Fig. 2 b).

Group C: Projections in the form of a rhombus, also with two-fold symmetry, divided into four domains by bands of stain extending from corner to corner. Length of edges: 80 – 90 Å, distance between opposite corners: 120 Å.

In addition many particles without a distinct substructure occur. Interestingly, most of the projections belong to group A. This suggests that a preferential orientation of the molecules with respect to the supporting film may exist.

Fig. 1 e shows the appearance of a pure preparation of 12.5S aminopeptidase fragments (M_r = 320 000). Clearly, most of the projections closely resemble particles of group A with three-fold symmetry. It seems, however, that the central hole of the 12.5S particles is more pronounced and the outline of the images is sharper.

6S aminopeptidase molecules which correspond to the dimeric form of the enzyme were isolated from density gradients run at pH 4. The electron micrographs (Fig. 1 f) show roughly circular or oblong particles not exceeding 60 Å in length. From our studies it is difficult to establish the actual shape of the dimers, however, since with molecules of this size the limits of the negative staining technique are reached.

As mentioned above, aggregation of aminopeptidase readily takes place in neutral solutions. Inspection of Fig. 1 d and Fig. 2 d – e provides some information on the mode of assembly of such aggregates.

1. Aggregates are formed by association of entire 22S molecules rather than by addition of further protomers to these particles. This is consistent with sedimentation experiments [4] which demonstrate distinct clearly separated peaks in the high-molecular-weight range.
2. Molecules participating in aggregates are never viewed all from the same side. If, for example, one of the components of a dimer is viewed along its threefold axis (*i. e.* belongs to group A) the other one will belong to group B (*cf* Fig. 2 d).
3. With trimers the angle between the lines connecting the centers of each pair of particles will be either 180°, 120° or 60°, respectively. In the first case linear aggregates are formed, in the second bent structures arise. An angle of 60° yields triangular aggregates. Examples for each type of trimers are shown in Fig. 2 e.

Usually the number of 22S particles involved in aggregates does not exceed 4 – 5. Regular arrays of higher numbers of particles were not observed in our experiments.

2. *The model*

A model for the quaternary structure of yeast aminopeptidase I which is consistent with the known facts and also meets the requirements of the 'symmetry concept' [6] is schematically depicted in Fig. 3. For the sake of simplicity in drawing the figure the dimeric subfragments were assumed to be spherical. A somewhat more realistic nonspherical subunit

shape was adopted for the construction of the three-dimensional models which are compared to actual particle projections in Fig. 2. The essential geometric features of both models are fully equivalent, however. This is illustrated by the lower part of Fig. 3 in which views of either model along the main axes are opposed to each other.

We assume that the active 22S molecule is composed of two stacked pseudo-trimeric rings. The smallest symmetric component of this structure is the dimeric 6S enzyme form made up from two protomers ($M_r = 53\,000$) joined in a symmetric fashion. A dodecameric protein assembled in this way has pseudo-D_3 symmetry with a single three-fold axis and three equivalent two-fold axes of symmetry.

Three different types of bonding are involved in the stabilization of the complex. The bonds connecting the two hexamers are the least stable as indicated by the spontaneous formation of 12.5S particles from 22S molecules. The bonds joining the three dimers of a hexamer are cleaved not above pH 4.5; to separate the two protomers forming the 6S particle denaturing agents or rather low values of pH are necessary.

The introduction of one further class of binding sites into such a particle allows the formation of aggregates with exactly the geometry observed with aminopeptidase aggregates. This is demonstrated by Fig. 2 d – e. The models shown were all constructed by joining model units along one of their two-fold axes and then rotating each two particles around this bond until their three-fold axes formed an angle of 120° with each other. The limited number of bond angles encountered in trimers and higher oligomers (60°, 120° or 180°) is a straightforward consequence of this type of connection.

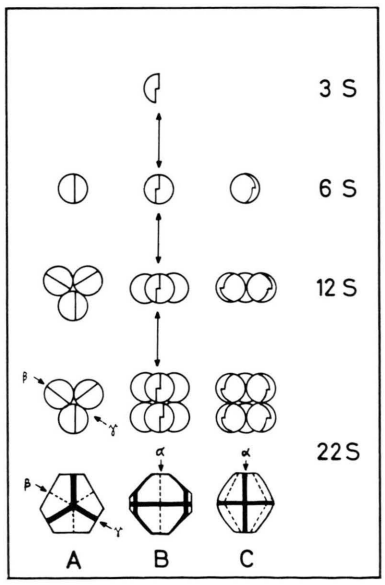

Fig. 3. Schematic representation of the model proposed for the quaternary structure of aminopeptidase I. The stepwise assembly of a 22S particle from 3S protomers is depicted. The left column contains views down the three-fold particle axis (α). The structures shown in the middle and right-hand columns are seen along a two-fold axis (β, γ). The bottom row illustrates how the space-filling models of Fig. 2 are related to the more general scheme based on spherical 6S units.

Discussion

Several experimental difficulties inherent to the negative staining method often prevent a meaningful interpretation of electron micrographs taken with proteins [2]. So the preparation of the specimens may cause artifacts by severe structural distortion of the particles. Secondly, the images observed with negatively contrasted molecules are projections of the three-dimensional structure on a plane to which both the top and the bottom of the particles contribute. Since the thickness of the stain is not known and may vary considerably within a given field rather different images may result from identical particles even if they are viewed from the same direction. This is especially the case if the molecules are randomly oriented relative to the supporting film.

None of these problems seems to interfere seriously with the interpretation of the electron micrographs presented above. First of all, very similar images were obtained with different stains and little change was observed upon variation of the staining procedure. Secondly, the particles are of a quite uniform appearance all over the specimens. A feature of yeast aminopeptidase, however, which is most favourable for the assessment of molecule structure by electron microscopy is the fact that well-defined subfragments of the enzyme may be isolated under non-denaturing conditions and thus are available for a separate examination. Finally, the existence of higher aggregates provides an independent means to judge the symmetry of the native enzyme molecules.

Summarizing the results given in the present as well as in the accompanying paper, one can state

that any reasonable model for the quaternary structure of the enzyme has to cover the following points:

1. The native aminopeptidase molecule (22S) is made up from 12 identical or closely related subunits.
2. Under physiological conditions this dodecamer is in equilibrium with a hexameric as well as a dimeric enzyme species. In addition it is capable of forming aggregates composed of entire 22S molecules.
3. The 22S particles are closed structures with a diameter of about 110 Å; they contain three-fold as well as two-fold axes of symmetry.
4. In the hexameric subfragment the three-fold axis is conserved. The dimensions of the 12.5S particle if viewed along this axis are similar to the dimensions of the 22S dodecamer.
5. The size and shape of isolated 6S fragments are similar to those of the smallest symmetric structure discernible in images of 12.5S and 22S particles.

It is now well documented that most of the multisubunit proteins examined so far, being made up of a single type of protomers, are assembled in such a way that all protomers occupy equivalent positions [6]. With dodecameric proteins only a few arrangements are possible which meet this requirement [7]. One of these is a stack of two hexagons which possesses dihedral (D_6) symmetry. Such a quaternary structure is found with glutamine synthetase from *E. coli* [8]; it is inconsistent, however, with several features of yeast aminopeptidase. Thus it does not account for the existence of stable dimers and the presence of a distinctly three-fold axis of symmetry in the molecule. Another type of arrangement which has to be considered with dodecamers includes several modifications of shell structures with cubic or tetrahedral symmetry. These cases have been discussed by Bowers *et al.* [9] as a possible model for the quaternary structure of L-aspartate decarboxylase from *Alcaligenes faecalis*.

This enzyme has a molecular weight similar to that of aminopeptidase I and a sedimentation coefficient of 19S. At slightly alkaline pH it dissociates into dimers which sediment at 5.8S. Hexameric fragments were not encountered. From simulation experiments a number of possible projections which could arise from negatively stained particles with tetrahedral shell structure were derived, some of which are not too different from images observed with yeast aminopeptidase. The application of the tetrahedral model to our enzyme fails, however, to account for distinctly triangular projections as a regular case; moreover, the dissociation of such a complex to give equal hexamers should not be a probable event.

Thus we give priority to the model outlined above, based on a pseudo-D_3 symmetry. The strongest support for this hypothesis comes from the experimentally established size and symmetry of the isolated fragments, which are in complete agreement with the features expected from the model. Although an unambiguous decision seems not to be possible at the moment, we assume that the pseudo-trimeric hexameric rings are stacked in an 'eclipsed' conformation as indicated in Fig. 3. A 'staggered' arrangement of the hexamers should lead to almost circular particle projections with a central hole if the thickness of the stain exceeds the diameter of the molecules. Triangular images would result only in the case of an exclusive bottom stain.

This work was supported by grants of the Deutsche Forschungsgemeinschaft.

[1] D. W. Darnall and I. M. Klotz, Arch. Biochem. Biophys. **166,** 651–682 [1975].
[2] R. H. Haschemeyer, Adv. Enzymol. **33,** 71–118 [1970].
[3] R. H. Haschemeyer and E. de Harven, Annu. Rev. Biochem. **43,** 279–301 [1974].
[4] G. Metz, Ruth Marx, and K.-H. Röhm, Z. Naturforsch. **32 c,** 929 [1977].
[5] G. Metz and K.-H. Röhm, Biochim. Biophys. Acta **429,** 933–949 [1976].
[6] J. Monod, J. Wyman, and J. P. Changeux, J. Mol. Biol. **12,** 88–118 [1965].
[7] N. M. Green, Protein-Protein Interactions (R. Jaenicke and E. Helmreich, eds.), p. 183 ff., Springer-Verlag, Berlin 1972.
[8] R. C. Valentine, M. B. Shapiro, and E. R. Stadtman, Biochemistry **7,** 2143–2152 [1968].
[9] W. F. Bowers, V. B. Czubaroff, and R. H. Haschemeyer, Biochemistry **9,** 2620–2625 [1970].