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Evidence for an Active Dimer of *Escherichia coli* β-Galactosidase

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β-Galactosidase (EC 3.2.1.23), prepared from strains ML 308 and K12 3300 of *Escherichia coli*, dissociated into an inactive monomer in the presence of Ag^+. When such a monomer preparation is treated with excess of thiol an enzymically active dimer is formed in addition to an active tetramer. It is suggested that Ag^+ may be of value in studies on other multimeric proteins as a mild dissociating agent.

In a previous investigation (Contaxis & Reithel, 1971) evidence was presented suggesting the existence of an active dimeric ($2n$) form of β-galactosidase from *Escherichia coli* K 12 and ML 308. In the present work we have succeeded in modifying the enzyme sufficiently by Ag^+ ion binding to allow the demonstration of an active dimer.

Homogeneous β-galactosidase, prepared as described by Contaxis & Reithel (1971), was dissolved in 50 mM-Tris–acetate, pH 9.2, at a concentration of about 2 μM-tetramer ($4n$). When such samples were held at 4°C and $AgNO_3$ was added at a ratio of Ag^+/tetramer ($4n$) of 30:1, nearly complete dissociation to an inactive monomeric form (n^{Ag}) could be observed by polyacrylamide-gel electrophoresis with the above buffer. A confirmatory set of experiments was done with β-galactosidase, in the same buffer, at a concentration of 2 mg/ml, and Ag^+/tetramer ($4n$) ratios of 14:1, 20:1, 27:1 and 64:1. After 2 h at 6°C these samples were examined with a Spinco model L ultracentrifuge at 6°C and 52000 rev./min. At the lower equal amounts of components with $s_{20,w}$ values of 6.3 and 16.2 S were seen. The larger ($4n$) species diminished in the higher-ratio mixtures until, at 64:1, only the 6.3 S component (n^{Ag}) was seen. At 6°C the monomeric species is stable for several days. This allowed the determination of molecular weight by high-speed sedimentation equilibrium by methods previously described (Contaxis *et al.*, 1973). Runs made at speeds at or near 15000 rev./min yielded $\bar{M}_w$ values of 119000, assuming a $\bar{v}$ value of 0.758 (Contaxis *et al.*, 1973).

After establishing conditions for observing the dissociation of β-galactosidase in the presence of Ag^+, we investigated the reassociation of the monomer (n^{Ag}) after removal of the Ag^+ with dithiothreitol. To an enzyme sample (3.7 mg/ml) was added $AgNO_3$ to obtain a ratio of 140:1 at 6°C; it was then left for 10 min. Then excess of dithiothreitol (dithiothreitol/Ag^+, 100:1) was added and the mixture was left for 3 h. Examination by gel electrophoresis in the same buffer as that used in the experiment (0.05 M-Tris–acetate buffer, pH 9.2) revealed three bands corresponding to the monomer (n) and the tetramer ($4n$) and an intermediate band, when stained with Coomassie Blue. When such gels were examined for enzyme activity by exposing them to 6-bromonaphthyl β-D-galactopyranoside and Diazo Blue B (Marchesi *et al.*, 1969), the intermediate band, as well as that corresponding to the tetramer, was observed to have catalytic activity. In a similar experiment, the dithiothreitol-treated species were examined in the Spinco model L ultracentrifuge. At 56000 rev./min and 18°C two peaks were visible. These two components proved to have $s_{20,w}$ values of 5.3 and 9.3 S. The first component (n^{DTT}) is a dithiothreitol-generated monomer, and the second, provisionally called ($2n^{DTT}$), was suspected to be a dimer. In other experiments where tetramer was present the $s_{20,w}$ value of enzymically active component ($4n^{DTT}$) was 14.4 S. An expected S value for a dimer obtained by using the relation $\left(\frac{M_1}{M_2}\right)^{\frac{2}{3}} = \frac{S_1}{S_2}$ is 9.1 S.

The presumed ($2n^{DTT}$) species was also formed when 2-mercaptoethanol was used in place of dithiothreitol. Since this species aggregates readily in the absence of thiol-group reagents it must be regarded as being metastable. As yet, attempts to obtain sufficient quantities for extensive characterization have been unsuccessful. Since a direct determination of molecular weight by sedimentation equilibrium was not immediately possible, but only the sedimentation constant, we sought to corroborate our assignment of the association state of this active unstable species by gel electrophoresis. This necessitated a suitable standard, which we obtained as follows. In preparing

β-galactosidase by using an affinity column (Cuatrecasas, 1970) we obtained eluates containing two compounds. One proved to be the expected active tetramer ($4n$), $s_{20,w}$ 16.4S. The other was an inactive species having $s_{20,w}$ 11.7S. The inactive smaller species was purified by density-gradient centrifugation in a glycerol gradient and also by a selective $(NH_4)_2SO_4$-extraction procedure. When the purified component was examined by high-speed sedimentation equilibrium at 7°C and 12000rev./min the $\bar{M}_w$ appeared to be 270000±10000, assuming a $\bar{v}$ of 0.758. Thus it appears that various amounts of an inactive dimer ($2n$) are normally present in β-galactosidase preparations. Moreover, it was noted that a component with the same electrophoretic mobility at various pH values was generated from homogeneous tetrameric ($4n$) β-galactosidase by treatment with high ratios of Ag^+ at pH values of 9.2 or higher. During electrophoresis it has a migration velocity corresponding to that of a dimer. The inactive dimer and the ($2n^{DTT}$) species have similar, but distinguishable, migration patterns. Addition of excess of Ag^+ to the inactive dimer led to aggregation. The addition of Ag^+ followed by dithiothreitol resulted in a mixture that formed an electrophoresis pattern with well-defined bands in the positions corresponding to monomer (n), dimer ($2n$) and tetramer ($4n$). Addition of dithiothreitol alone to the inactive dimer (in 0.1 M salt) resulted in a mixture that yielded an electrophoresis pattern with components corresponding to components ($2n$) and ($4n$), the latter being slightly active. Thus the $s_{20,w}$ and migration values obtained for the inactive dimer and products derived from it were those we had come to associate with β-galactosidase. We considered it reasonable to discard the possibility that the inactive protein was totally unrelated and fortuitously retained on the column. The reactivation observed was not conclusive since the weak activity possibly could have derived from a trace of tetramer that was capable of being reactivated.

Experiments (Contaxis & Reithel, 1971) showing dissociation of tetrameric ($4n$) β-galactosidase to ($n^{glycerol}$) species afforded the opportunity to compare it with component (n^{Ag}). The two forms of monomer were not easy to distinguish by gel electrophoresis, but the $s_{20,w}$ values differed. As stated above, the value for component (n^{Ag}) is 6.3S whereas that for component ($n^{glycerol}$) is 5.1S. If component ($n^{glycerol}$) is prepared and then exposed to Ag^+, under the same conditions as those used to obtain component (n^{Ag}), a form ($n^{glycerol,\,Ag}$) is obtained with $s_{20,w}$ 6.2S. A monomer that is formed by simple dilution in buffer (n^{dil}) has an $s_{20,w}$ value of 4.6S, which suggests extensive unfolding (Contaxis *et al.*, 1973). All $s_{20,w}$ values reported here were measured in 0.05 M-Tris–acetate buffer, pH9.2, at 52000rev./min and 6°C.

Most procedures that facilitate the dissociation of multimeric proteins result in unfolding. Hence lack of activity by a dissociated form obtained by such procedures cannot be uniquely ascribed to dissociation. The dissociation of β-galactosidase in glycerol (Contaxis & Reithel, 1971) resulted in a monomer that was not unfolded but nevertheless had no catalytic activity. There remained the possibility that a single (n)–(n) interaction would supply the conformational changes associated with activity, and hence a tetramer ($4n$) need not be the smallest active species. In the present investigation it has been shown that Ag^+ binding results in conformational changes, indicated by the sedimentation data, and that the dissociated monomers, on removal of the Ag^+, can reassociate to an active dimer. It seems clear that only one subunit–subunit interaction is required for enzyme activity, although this species associates readily to a tetramer in the usual buffers.

Bivalent mercury has been used successfully to potentiate the dissociation of multimeric proteins (Vanaman & Stark, 1970), but a similar use of univalent silver has not been reported. In a companion study on urease (Kaneshiro, 1975) the results of the binding of Ag^+ were compared with those of the binding of Hg^{2+} for both urease and β-galactosidase. Although it is highly likely that both Ag^+ and Hg^{2+} bind only to thiol groups at the high dilutions used, the association–dissociation effects differed. In the present investigation there was no evidence to suggest that unfolding accompanied dissociation or that it was irreversible. It is suggested that the judicious use of Ag^+ may be applicable in other investigations of multimeric proteins. As a working hypothesis it has been assumed that neither glycerol nor Ag^+ is a causative agent in dissociation. Rather, it is assumed that they affect the conformation of that part of the protein domain involved in (n)–(n) interaction to prevent reassociation.

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