KINETICS OF THE DECAMER - DIMER DISSOCIATION OF ARGININE DECARBOXYLASE

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SUMMARY

The kinetics of decamer to dimer dissociation of the inducible arginine decarboxylase of *Escherichia coli* B have been examined by reacting the single newly available sulfhydryl residue with 5,5'-dithiobis-(2-nitrobenzoic acid). The empirical rate law is first order with respect to this reagent and to the protein concentration, and is saturating with respect to $[Na^+]^2$ and $1/[H^+]^2$; *i.e.*, the rate of dissociation increases with increasing Na^+ and decreasing H^+. The results suggest that the decamer - dimer transition is sequential, occurring in five steps, and that two protons must ionize and two Na^+ ions bind at each step.

The generally available techniques for the study of enzyme dissociation reactions provide information only about the equilibria involved; the actual mechanisms usually remain unknown. The inducible arginine decarboxylase of *Escherichia coli* B, for example, has been shown to dissociate from the decamer (mol. wt. 820,000) to the dimer (mol. wt. 160,000) as the pH increases and the concentration of monovalent cations decreases (1). Schematically,

$$E_{10} \rightleftharpoons 5\ E_2 + n\ Na^+ + n\ H^+$$

Dissociation proceeds to completion at Na^+ concentrations less than .04 M and pH values greater than 6.5.

The equilibrium arrows in this expression, however, are a black box; detailed information about the dissociation process itself is not available. Since intermediate species are not observed in the analytical ultracentrifuge during dissociation, and are observed during association only when a competitive inhibitor is present, this technique cannot be used to determine whether they occur as transient species, *i.e.*, whether dissociation is sequential or concerted. The expression above suggests that the Na^+ dependence is due to a

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direct ionization; it could also be due to an effect of salt concentration on the strength of intersubunit bonds. In addition, protons could be produced independently from each subunit, yielding 10 or some multiple of 10 for each decamer that dissociates, or they might be produced in a way that indicates co-operation between dissociating subunits. These questions can be answered by studying the mechanism of the dissociation reaction.

Kinetic techniques cannot ordinarily be used to study dissociation mechanisms since proteins usually do not undergo a chemical change when they dissociate. The dimer of arginine decarboxylase, however, reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)[1] while the decamer does not (2); one sulfhydryl residue per subunit (two per dimer) is exposed during dissociation. By coupling the dissociation reaction to the production of thionitrobenzoate from DTNB and following the increase in absorbance at 412 nm (3), it is possible to examine the kinetics of arginine decarboxylase dissociation.

METHODS

Arginine decarboxylase was purified as previously described (2); protein determinations were performed in a Beckman DB spectrophotometer using an extinction coefficient, $E^{1\%}_{280} = 15.7$ (4). The enzyme was assayed in 0.2 M Na acetate buffer, pH 5.2, containing 0.15 M L-[U-^{14}C]arginine and .05 mM pyridoxal-P, using the technique of Morris and Pardee (5). The starting material for each study was at least 95% decamer, as shown in a Beckman model E analytical ultracentrifuge.

Rates of dissociation were measured at 20° by following the formation of the thionitrobenzoate anion at 412 nm in a Bausch and Lomb Spectronic 505 spectrophotometer equipped with a Heathkit recorder, Model EUW-20A, and a water jacketed cell compartment. After exhaustive dialysis against the appropriate low concentration (1 - 12 mM) of Na phosphate, pH 5.2, arginine decarboxylase was added to a spectrophotometer cell together with a solution containing the required amounts of DTNB and additional Na phosphate, also at pH 5.2. Dissociation was begun by increasing the pH with NaOH. The blank contained all reagents except arginine decarboxylase. Initial rates were estimated graphically and the corresponding rate constants were obtained by dividing by the protein concentration.

RESULTS

Rate equations for particular dissociation mechanisms can be readily derived when the rates measured are initial rates and the concentrations of substances other than the various enzyme species can be assumed to be con-

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[1]The abbreviation used is DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

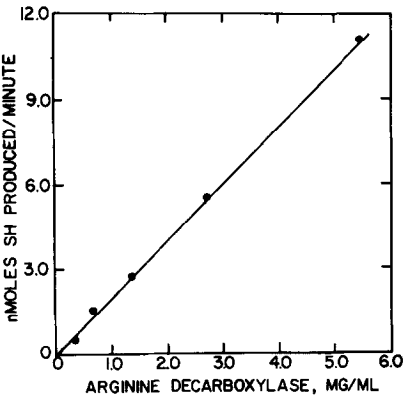

Figure 1. Rate of dissociation as a function of the concentration of protein. Final concentrations: DTNB, 0.78 mM; Na^+, 20 mM; pH, 7.2; μ, 28 mM.

stant. The derivations are analagous to those for enzyme reactions and produce rate equations of the type

$$\text{rate} = \frac{R_{max}}{1 + \frac{K_D}{[DTNB]^n} + \frac{K_H}{[H^+]^n} + \frac{K_N}{[Na^+]^n} + \ldots .},$$

where the number and type of cross-terms depend on the mechanism used. The exponents, n, are positive if the substance is a reactant and negative if it is a product; their magnitude is determined by the number of molecules involved in each dissociation event. Since initial rates are measured, only the first dissociation step is observed. For a simultaneous mechanism, this will be the production of five dimers from a single decamer; for a sequential mechanism, it is the production of the open decamer from the closed form.

Although the enzyme concentration does not appear in the above equation, it is clear that the rate of dissociation should decrease with the first power of the protein concentration unless the decamers interact. Figure 1 shows a simple first order dependence. This indicates that any dissociation mechanism inferred at the concentrations used here should be equally valid at physiological concentrations, which are much lower.

The dependence of the rate of dissociation on DTNB concentration is shown in Figure 2. For initial rates, only the reaction of the first sulfhydryl group exposed during dissociation should be kinetically visible, giving an empirical rate law that depends on the first power of the DTNB concentration. Figure 2 shows that this is true, and also that the reaction does not saturate

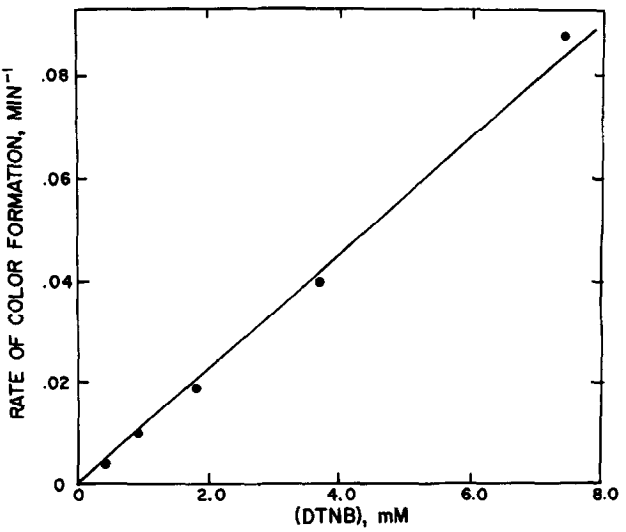

Figure 2. Rate of dissociation as a function of the concentration of DTNB. Final concentrations: arginine decarboxylase, 2.3 mg/ml; Na^+, 20 mM; pH, 7.2; μ, 31 - 34 mM.

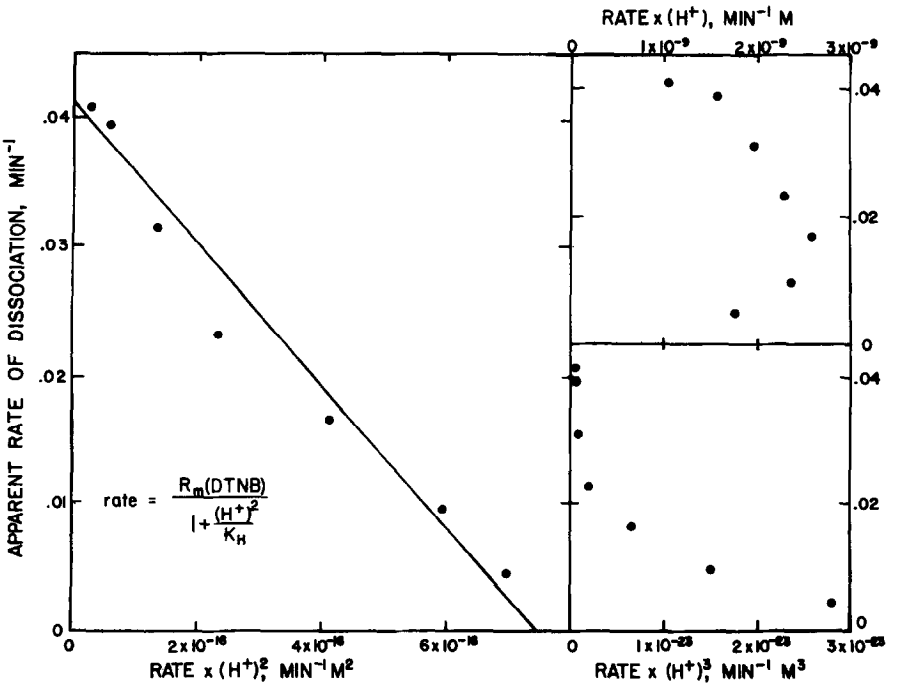

Figure 3. Rate of dissociation as function of the pH; measurements were made at pH 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, and 7.6. Final concentrations: arginine decarboxylase, 3.8 mg/ml; Na^+, 12 mM; DTNB, 0.93 mM; μ, 14 - 17 mM. The empirical rate law derived from the data in Figures 2 and 3 is shown in the lower left corner.

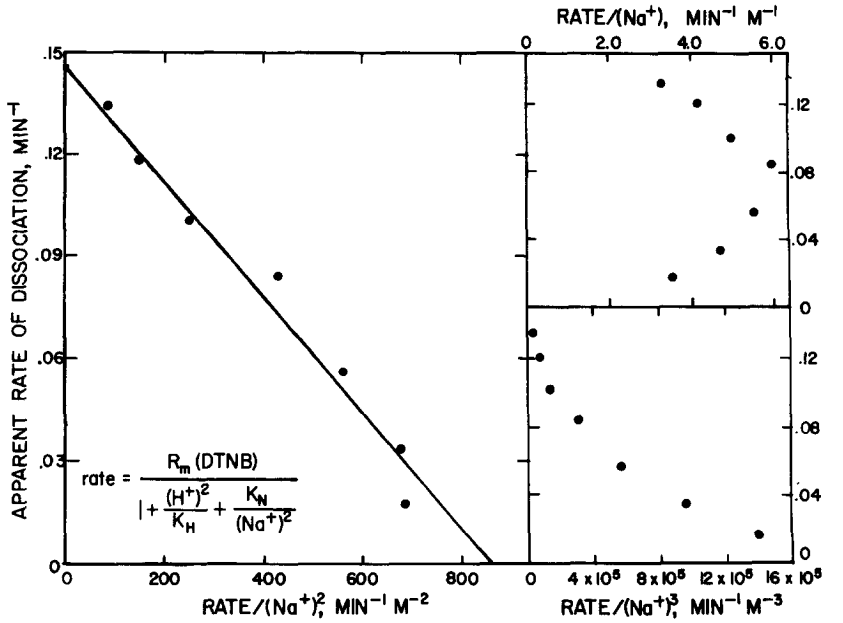

Figure 4. Rate of dissociation as a function of the concentration of Na^+. Final concentrations: arginine decarboxylase, 3.6 mg/ml; pH, 7.2; DTNB, 0.92 mM; μ, 5 - 80 mM. The empirical rate law derived from the data in Figures 2, 3 and 4 is shown in the lower left corner.

at the DTNB concentrations chosen. Kinetically, this means that the DTNB reaction is rate limiting; all steps prior to it can be assumed to be in equilibrium. Rate equations for particular mechanisms will now have the form

$$\text{rate} = \frac{R_{max}[\text{DTNB}]}{1 + \frac{K_H}{[H^+]^n} + \frac{K_N}{[Na^+]^n} + \ldots .},$$

where the definitions of K_H, K_N and the cross-terms differ from the cases where the DTNB concentration nears saturation.

Rates of dissociation as a function of pH and Na^+ concentration are shown in Figures 3 and 4; the plots are similar to Eadie plots. In both cases, the dependence is clearly second order, indicating that two molecules of each substance must be involved in the first dissociation step. Since the rate of dissociation increases as the H^+ concentration decreases, two protons must be produced; this is consistent with the equilibrium data previously obtained (1). For Na^+, however, the rate of dissociation increases as the concentration increases, indicating that two molecules bind during each step. This is the

reverse of the result obtained in the ultracentrifuge, suggesting that another step, involving the release of Na^+, must occur after the DTNB reaction, when it would be kinetically invisible. The spectrophotometric results confirmed the equilibrium results obtained in the ultracentrifuge, at least qualitatively; even though the decamer began to dissociate more rapidly at high Na^+ concentrations, dissociation ceased much earlier.

DISCUSSION

From Figure 3 it can be calculated that the pH where the rate of dissociation is half-maximal is 6.9, and from Figure 4, the half-maximal Na^+ concentration is 13 mM. Although these numbers cannot be interpreted simply, since the other variables were not saturating, their order of magnitude indicates that dissociation can occur under physiological conditions. The observed rate constants support this conclusion even though DTNB is limiting; the half-time of the extrapolated maximum rate in Figure 4 is five minutes.

The results also suggest that the first dissociation step requires the ionization of two protons and the binding of two Na^+ ions. Considering that the DTNB reaction must occur last, there are 14 distinct combinations of dissociation, H^+ ionization and Na^+ binding (6); this includes Theorell-Chance mechanisms and mechanisms where protons inhibit dissociation by forming dead-end complexes instead of products. For each combination, dissociation can occur either simultaneously or sequentially. However, all but five of the simultaneous cases produce a fifth or tenth order dependence on Na^+ or H^+ or both, and the complete rate equations for two of these cases do not fit the quantitative results in Figures 3 and 4. Although simultaneous mechanisms are not completely ruled out, the results strongly suggest that dissociation proceeds sequentially, step by step, rather than all at once.

Experiments where Na^+ is varied at fixed pH values, or DTNB at fixed levels of Na^+, etc., can be used to distinguish between the various dissociation mechanisms even though their effectiveness is reduced by a rate limiting DTNB reaction. This has not been attempted with the equipment used here because the applicability of the rate equations depends entirely on the measurement of initial rates. Since the mixing of reagents and the initial spectrophotometer response requires at least 15 seconds, a time which is significant with respect to many of the rates measured here, these results must be treated as preliminary. Using them as a baseline, I am now performing detailed experiments, designed to define the actual mechanism of dissociation, in a stopped-flow apparatus.

REFERENCES

1. Boeker, E. A., and Snell, E. E. (1968), J. Biol. Chem. 243, 1678.

2. Boeker, E. A., Fischer, E. H., and Snell, E. E. (1969), J. Biol. Chem. 244, 5239.

3. Ellman, G. L. (1959), Arch. Biochem. Biophys. 82, 70.

4. Blethen, S. L., Boeker, E. A., and Snell, E. E. (1968), J. Biol. Chem. 243, 1671.

5. Morris, D. R., and Pardee, A. B. (1965), Biochem. Biophys. Res. Commun. 20, 697.

6. Boeker, E. A., manuscript in preparation.