Crystallization of NADP-Specific Isocitrate Dehydrogenase

Abstract. *The first crystalline preparation of isocitrate dehydrogenase specific for nicotinamide adenine dinucleotide phosphate has been obtained with enzyme isolated from* Escherichia coli. *Scanning electron microscopy was employed to elucidate the structure of the crystals, which were found to exist as regular octahedrons ranging in size from 5 to 90 micrometers.*

Two distinct types of isocitrate dehydrogenase occur widely in nature. One type possesses a specific coenzyme requirement for nicotinamide adenine dinucleotide (NAD) (E.C. 1.1.1.41), while the other is specific for nicotinamide adenine dinucleotide phosphate (NADP) (E.C. 1.1.1.42). In mammalian cells and yeast, the NAD-dependent enzyme is generally associated with the mitochondria, while the NADP-dependent form is found in both the mitochondria and the cytoplasm (*1*). *Escherichia coli* and most other bacteria contain only the NADP-specific form of the enzyme (*2*).

Isocitrate dehydrogenase occupies a central position in the tricarboxylic acid cycle of terminal oxidation; it is also important in biosynthetic pathways as a mechanism for providing the cell with reducing equivalents in the form of reduced pyridine nucleotides. In systems where both the NAD- and NADP-specific forms of the enzyme occur, the activity of the former appears to be of primary importance in the catalysis of isocitrate oxidation in the tricarboxylic acid cycle. This NAD-specific form is allosterically regulated by the intracellular levels of adenosine diphosphate (ADP) (*3*). Detailed knowledge of the regulatory properties of the NADP-specific enzyme is lacking.

Electrophoretically pure NADP-dependent isocitrate dehydrogenase was isolated from *E. coli* as previously described (*4*). The enzyme was subsequently crystallized according to the method of Jakoby (*5*) using 12.7 m*M* citrate-phosphate buffer, *p*H 5.5, containing 2 m*M* $MgCl_2$. Crystals were obtained at ammonium sulfate concentrations of 65, 62, and 60 percent salt saturation.

The specific activity (micromoles of NADP reduced per minute per milligram of protein at 340 nm and 25°C) of the crystalline preparation was 48, whereas the specific activity of the uncrystallized, electrophoretically pure enzyme was 27. This suggests that only the catalytically active form of the enzyme is crystallized.

The crystals were examined with the light microscope (Fig. 1). The low-power objective provided good depth of field and resolution, but the degree of magnification was unsatisfactory (Fig. 1A). When the magnification was increased, difficulties were encountered in obtaining a focused image of the three-dimensional structures (Fig. 1B). On the basis of the light microscopic examination, we postulated that the native enzyme crystals exist in the form of regular octahedrons (or tetrahedral bipyramids) ranging in size from 5 to 30 μm.

Hoping to obtain higher magnification and better resolution, we made an effort to examine the crystals with a scanning electron microscope. In the preparation of the crystals for examination with this instrument, two technical problems were encountered. First, it was observed that the crystalline structure was destroyed as a result of the drying process. A similar observation has been reported by Inoue *et al.* (*6*) working with tropomyosin crys-

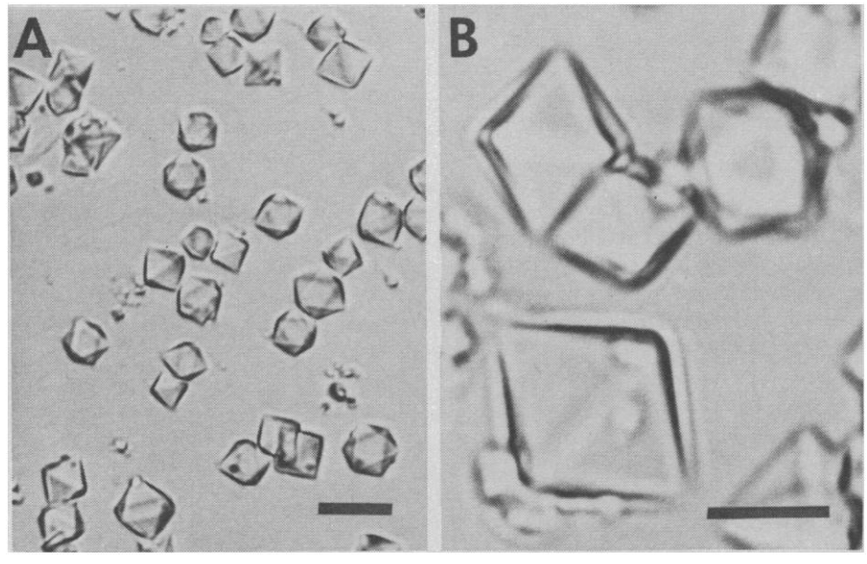

Fig. 1. Light microscope photomicrographs of NADP-specific isocitrate dehydrogenase crystals. Photomicrographs taken with (A) low-power objective (scale, 10 μm) and (B) high-dry objective (scale, 5 μm).

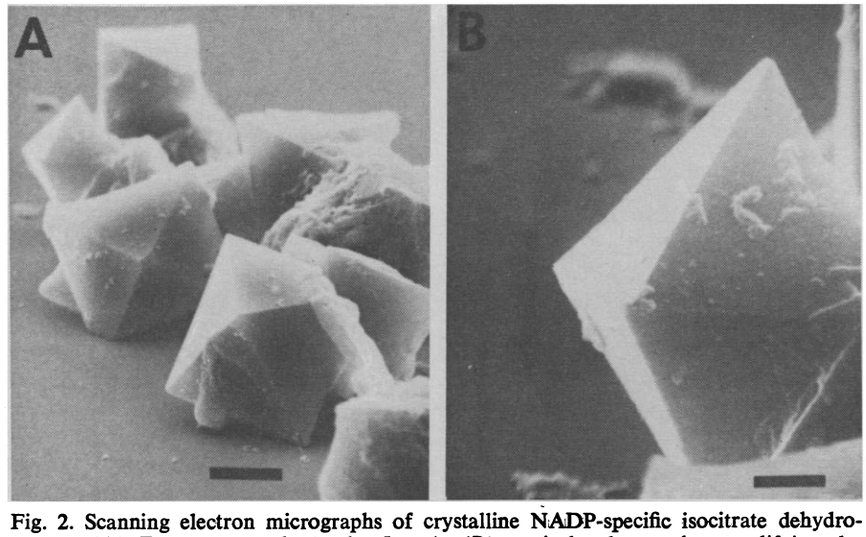

Fig. 2. Scanning electron micrographs of crystalline NADP-specific isocitrate dehydrogenase. (A) Enzyme crystals (scale, 5 μm); (B) an isolated crystal exemplifying the characteristic octahedral geometry (scale, 1 μm).

tals. Second, the ammonium sulfate used in the suspension medium to maintain the integrity of the crystals and to prevent redissolution of the protein crystallized on drying. We therefore sought a treatment which would impart mechanical strength to the crystals and simultaneously prevent dissolution of the protein in the volatile buffers required for electron microscopy.

Glutaraldehyde has been successfully used as a fixative in the preparation of specimens for electron microscopy (*7*). Quiocho and Richards (*8*) have shown that single crystals of carboxypeptidase A fixed with this reagent have x-ray diffraction patterns very similar to those of the native crystals. These authors have also demonstrated a marked increase in the mechanical stability of crystals prepared for x-ray diffraction studies following treatment with glutaraldehyde.

We therefore fixed the isocitrate dehydrogenase crystals by adding glutaraldehyde to the 60 percent ammonium sulfate suspension of crystals to make the resulting solution 3 percent with respect to this fixative. The suspension was maintained at 4°C for 12 hours. The fixed crystals were recovered by centrifugation at 500*g* for 5 minutes at 4°C, the supernatant was siphoned off, and the crystals were resuspended in 20 m*M* ammonium acetate, *p*H 6.9. This suspension was then centrifuged at 500*g* for 5 minutes at 4°C, the supernatant was removed, and the crystals were again suspended in the ammonium acetate buffer. This procedure was repeated three times. After the final centrifugation, the washed crystals were suspended in 1 ml of the 20 m*M* ammonium acetate buffer.

The treatment of the isocitrate dehydrogenase crystals with this cross-linking agent produced no changes in crystalline morphology that were detectable with the light microscope. The fixed crystals were found to be insoluble in both 20 m*M* ammonium acetate, *p*H 6.9, and distilled water. However, when the ionic strength of the suspension medium was lowered after fixation with glutaraldehyde, the crystals were observed to clump.

The buffered crystal suspension was dispersed on glass cover slips broken to fit scanning specimen stubs and dried at room temperature (23°C). The fixed material was dried with no apparent disruption of the morphology. The cover slips were then mounted on specimen stubs with silver paint, and vapor-coated with carbon followed by gold while they were rotating on a motorized platform. The specimens were viewed with a Cambridge Stereoscan scanning electron microscope at an accelerating voltage of 20 kv.

Examination of the enzyme crystals with the scanning electron microscope provides definitive proof of their octahedral structure. Figure 2A is an electron micrograph of the protein crystals under low magnification. Note that the effective magnifications of the enzyme crystals in Figs. 1B and 2A are approximately the same; yet, the resolution provided by the scanning electron microscope is vastly superior. The amorphous material acting as a cementing substance is assumed to be uncrystallized enzyme which has been cross-linked to the crystals by the glutaraldehyde. Figure 2B is a higher magnification of a small, individual crystal which exemplifies the octahedral morphology postulated from the light microscopic examinations.

This represents the first report of a successful crystallization of isocitrate dehydrogenase, either NAD- or NADP-specific, from any source. Further, to the best of our knowledge, it is the first investigation of the structure of an enzyme crystal by scanning electron microscopy. The techniques described provide a general method for the examination of fragile enzyme crystals with the scanning electron microscope. These crystals, which do not lend themselves to study with either the light microscope or the transmission electron microscope because of their three-dimensional nature, are well suited for observation with the scanning electron microscope. This instrument provides magnification and resolution without loss of the depth of field required for detailed examination of crystals of this nature.

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References and Notes

1. G. D. Greville, in *Citric Acid Cycle*, J. M. Lowenstein, Ed. (Dekker, New York, 1969), p. 25.
2. J. M. Lowenstein, in *Metabolic Pathways*, D. M. Greenberg, Ed. (Academic Press, New York, 1967), vol. 1, p. 146; G. W. E. Plaut, in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myerbach, Eds. (Academic Press, New York, 1963), vol. 7, p. 105; T. E. Rogland, T. Kawaski, J. M. Lowenstein, *J. Bacteriol.* **91**, 236 (1966).
3. R. F. Chen, D. Brown, G. W. E. Plaut, *Biochemistry* **3**, 552 (1964).
4. H. C. Reeves, G. O. Daumy, C. C. Lin, M. Houston, *Biochim. Biophys. Acta* **258**, 27 (1972).
5. W. B. Jakoby, *Anal. Biochem.* **26**, 295 (1968).
6. S. Inoue, J. Hardy, G. H. Counsineau, A. K. Bal, *J. Ultrastruct. Res.* **29**, 350 (1969).
7. D. D. Sabatini, K. Bensch, R. J. Barrnett, *J. Cell Biol.* **17**, 19 (1963).
8. F. A. Quiocho and F. M. Richards, *Proc. Nat. Acad. Sci. U.S.A.* **52**, 833 (1964).
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Common Mechanism for Repellents and Attractants in Bacterial Chemotaxis

Abstract. *The migrational response of* Salmonella typhimurium *away from compounds such as phenol, indole, acetic acid, and leucine occurs because the bacteria tumble less frequently while* descending *gradients of repellents. This contrasts with their response of tumbling less frequently while* ascending *gradients of attractants. The results of competition experiments suggest that repellents, like attractants, operate through specific receptors, and the algebraic additivity experiments indicate that repellents and attractants utilize a common memory mechanism for taxis.*

The migration of bacteria toward attractants and away from repellents was observed in the last century by Pfeffer and Engelmann (*1*). Since that time the response to attractants has been documented extensively by Adler and co-workers (*2, 3*) and more recently by Berg and Brown (*4*) and in our laboratory (*5, 6*). However, except for the finding by Lederberg (*7*) of negative phenol taxis and the *p*H and inorganic ion studies by Doetsch and co-workers (*8*), repellents have received little attention. The recent development of new techniques (*5, 6*) has facilitated the study of repellents as well as attractants. Moreover, the finding that attractant gradient sensing employs specific receptors (*2, 9, 10*) and a memory mechanism