Impaired Granulocyte Superoxide Production and Prolongation of the Respiratory Burst Due to a Low-Affinity NADPH-Dependent Oxidase

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A 7-yr-old girl with self-limited pulmonary aspergillosis was found to have a defect in granulocyte superoxide production. Her cells produced superoxide at 3% of control rates in response to phorbol myristate acetate (PMA) and opsonized zymosan. Lag times for O₂ production were normal with PMA, opsonized zymosan, and concanavalin-A stimulation. Her granulocyte membranes depolarized in response to all of these stimuli. Superoxide produced by podosomes and a particulate fraction demonstrated an enzyme activity with a normal maximal velocity but a decreased affinity for NADPH. NADH-dependent superoxide production by particles was similar with patient and control material. The duration of superoxide production was prolonged in the patient’s intact granulocytes and in the particulate fractions from her cells. Bacterial killing by the patient’s granulocytes was initially low, but approached control values after 90 min of incubation. These results are explained by an enzyme activity that has a decreased affinity for its substrate and a decreased rate of inactivation. Family studies indicate an autosomal recessive mode of inheritance.

DEFENSE AGAINST BACTERIAL and fungal infections depends on the action of phagocytic leukocytes, which ingest and kill potentially pathogenic organisms. Granulocytes and monocytes from patients with chronic granulomatous disease (CGD) have impaired microbicidal capacity because of their inability to generate metabolites of oxygen that are toxic to bacteria and fungi.

Intact neutrophils and monocytes from patients with CGD do not depolarize and do not produce superoxide. These defects are associated with major susceptibility to invasive bacterial and fungal infection. Although the majority of cases of CGD are inherited in an X-linked recessive manner, these disorders are clinically severe and frequently fatal at a young age. We describe a patient with a disorder of the respiratory burst that appears to be inherited in an autosomal recessive manner. The oxidase of the patient’s phagocytes is normally activated, depolarization is demonstrable, but severely reduced levels of superoxide are produced. The oxidase activity in membrane preparations has an apparent altered affinity for reduced NADPH. A prolonged respiratory burst occurs in both intact cells and membrane preparations. A previously reported patient with a high Kₘₐₚ for NADPH has a disorder that appears to be inherited in an X-linked fashion. Ingested microorganisms are eventually killed at levels approaching those of normal cells despite failure of killing after short incubation times.

This child’s only clinical problem was bronchopulmonary aspergillosis, which was not severe and resolved without specific antifungal therapy. Her brother died of invasive aspergillosis shortly after being given corticosteroids for presumed allergic bronchopulmonary aspergillosis. Although phagocyte function was not examined during life, he had multiple granulomata at autopsy. The clinical courses of these siblings imply that even severely defective phagocyte function is capable of controlling invasive organisms as long as other immunologic functions and the inflammatory process remain intact. These and similar cases suggest the wisdom of documenting that a patient is immunologically competent before beginning immunosuppressive therapy in the face of active infection.

CASE REPORT

The patient, now 7 yr old, was first referred for evaluation at 2 yr of age. Her brother had experienced multiple episodes of acute exacerbation of a chronic interstitial pneumonitis from the age of 4...
yr. Two weeks before his death at age 14, he was begun on systemic hydrocortisone, with transient improvement of his respiratory symptoms, followed by development of respiratory failure. Amphotericin was begun after an open lung biopsy showed invasive aspergillosis. At autopsy, granulomata were found in skin, lymph nodes, and the gastrointestinal tract, raising the suspicion of immunodeficiency for the first time.

His sister developed fever and tachypnea at age 2 yr. An open lung biopsy performed because of a diffuse interstitial pneumonitis showed multiple granulomata, with fibrosis, necrosis, and giant cells. Fragments of fungal hyphae consistent with Aspergillus in varying stages of degeneration were found on silver stain. Bacterial and fungal cultures of the specimen were negative.

She was discharged to live with relatives away from her rural farm home for a year. She improved clinically without antifungal therapy. She has mild restrictive lung disease. Both clinical and radiologic improvement were very gradual, but she was asymptomatic within 6 mo, and her chest radiograph was normal after 3 yr. She has had no boils, no deep tissue infections, no hepatosplenomegaly or adenopathy. Cutaneous lesions become infected easily, and heal slowly, with granuloma formulation. She is not on prophylactic antibiotics, although she does receive careful attention to local hygiene and systemic antibiotics for minor infections. Growth and development have been normal. Both parents and a sister are clinically well, and there is no history of undue susceptibility to infection in other relatives.

MATERIALS AND METHODS

Cytochrome-C type VI, superoxide dismutase, NADPH, NADH, nitroblue tetrazolium, and zymosan were purchased from Sigma Chemical Company, St. Louis, MO. Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Chemical Company, Brewster, NY; dimethyl sulfoxide from Fisher Scientific Company, Pittsburgh, PA; Ficoll-Hypaque from Pharmacia Fine Chemicals, Piscataway, NJ. These and all other reagents were obtained at the highest level of purity and used without further purification. All surfaces in contact with cells were plastic or siliconized glass, except for spectrophotometer cuvettes.

Peripheral blood granulocytes and monocytes were collected in acid-citrate-dextrose and purified by Ficoll-Hypaque sedimentation. 1 T lymphocytes were removed from the mononuclear cell layer by rosette formation, leaving a preparation consisting of 65% monocytes by nonspecific esterase stain. Contaminating red cells were removed by hypotonic lysis with 0.87% NH4Cl. Washed cells were suspended in Krebs-Ringer phosphate (KRP) buffer or phosphate-buffered saline (PBS) with 5 mM glucose.

Quantitative ingestion of endotoxin-coated oil droplets opsonized with complement and simultaneous quantitative reduction of nitro-blue tetrazolium (NBT) were performed as described. 11 To assess NBT reduction by individual cells, adherent neutrophils and monocytes were exposed to 1 µg PMA/ml or 15 µg/opsonized zymosan particles/ml in the presence of 1% NBT in KRP on glass coverslips at 37°C for 15 min, counterstained with 1% safranin for 1 min, and examined for microscopic deposits of blue formazan dye. Cells were scored as 0–4 plus.

Killing of S. aureus was tested in a fluorochrome microassay in which ingested organisms are scored as viable or nonviable on the basis of staining with acridine orange. 14 Killing was assessed after 15, 30, 60, and 90 min of incubation.

Measurement of superoxide-dependent cytochrome-C reduction (ΔA560) was performed in a Perkin-Elmer Model 571 double-beam spectrophotometer. 15 Sample and reference cuvettes contained 2.5 × 10⁶ PMN and 50 nmole ferricytochrome-C in KRP with 5 mM glucose. Reference cuvettes contained 10 µg superoxide dismutase as well. At zero time, either 3 mg opsonized zymosan or 1 µg PMA was added. 16 Total volume was 1.0 ml. Assays were performed at 22°C and 37°C, and the rate of superoxide production calculated from the linear rate of absorbance change and the molar extinction coefficient for this reduction, 21,000. 17 The lag time was calculated from the intercept of the back-extrapolated linear portion of the curve with the preactivation baseline of zero absorption change.

Superoxide production by membrane-rich fractions was assessed in preparations made from intact cells stimulated for 4 min with 1 ng PMA/ml. Podosomes were prepared by an 8–10 sec immersion of warmed, PMA-stimulated neutrophils (10⁴ PMN/ml KRP) in a sonicator water bath, followed by vigorous mixing and cooling to 0°C. 18 Cell bodies were removed by centrifugation at 100 g for 5 min at 4°C. The supernatant was then spun at 12,000 g for 10 min, and the pelleted podosomes suspended at 1–2 mg protein/ml in 0.34 M sucrose. Particulate fractions were prepared from neutrophils stimulated with 1 µg PMA/ml for 4 min at 37°C. 19 Pelleted cells were suspended at 10⁶ cells/ml in cold 0.34 M sucrose, and homogenized on ice for 5–10 min in a glass-Teflon homogenizer until approximately 98% cell disruption had been achieved. Large fragments were removed by centrifuging the homogenate at 200 g for 10 min at 4°C. The supernatant was centrifuged at 27,000 g for 20 min at 4°C. The pellet or particulate fraction was resuspended in a Dounce homogenizer to a volume of 0.34 M sucrose, equivalent to 10⁴ PMN/ml.

The kinetics of superoxide generation by freshly prepared PMA-stimulated podosomes and particulate fraction was assayed by SOD-inhibitable cytochrome-c reduction in the presence of reduced pyridine nucleotides. Cytochrome-c (50 nmole), NaCN (0.5 µmole), and NADPH or NADH in varying concentrations were added to both the sample and the reference cuvettes in 0.9 ml 0.05 M potassium phosphate buffer, pH 7.5. The reference cuvette also contained 20 µg SOD. The podosomes or particulate fraction (0.1 ml) were added to both compartments, and continuous measurement of SOD-inhibitable cytochrome-c reduction was recorded at 37°C. For some experiments, an additional 50 nmole cytochrome-c was added after the reaction had ceased to determine whether the substrate had been exhausted.

Changes in membrane potential were assessed by monitoring the fluorescence of potential-sensitive probes in the presence of neutrophils exposed to activating stimuli. For studies with opsonized zymosan and PMA, the dye di-S-C₅(5) was utilized. 14 Concanavalin-A-induced depolarization was measured utilizing the dye di-O-C₅(3). 20 Fluorescence of potential-sensitive probes in the presence of neutrophils exposed to activating stimuli. For studies with opsonized zymosan and PMA, the dye di-S-C₅(5) was utilized. 14 Concanavalin-A-induced depolarization was measured utilizing the dye di-O-C₅(3). 20 Fluorescence of potential-sensitive probes in the presence of neutrophils exposed to activating stimuli. For studies with opsonized zymosan and PMA, the dye di-S-C₅(5) was utilized. 14 Fluorescence was monitored continuously in thermostatted, stirred cuvettes in a Perkin-Elmer Model 650-10 spectrophotometer. The probes were added from stock solutions in ethanol, such that the final concentration of ethanol was 0.2%. Di-S-C₅(5) was used at 2 × 10⁻⁴ M, with an excitation beam of 620 nm, an emission wavelength of 670 nm, and 2 × 10⁶ PMN in the cuvette. Di-O-C₅(3) was used at 5 × 10⁻⁴ M, with an excitation beam of 470 nm, an emission wavelength of 500 nm, and 5 × 10⁶ PMN in the cuvette. Concanavalin-A was used at a concentration of 100 µg/ml in the presence of 5 µg cytochalasin-B/ml. PMN was used at 1 µg/ml, and opsonized zymosan at 1 mg/ml. Proteins were extracted from zymosan particles with detergent and alkali treatment by a modified method of Law. 23 Addition of up to 1.0 mg/ml of these particles to di-S-C₅(5) had no effect on the fluorescence of the dye. 24 In contrast, addition of crude zymosan particles to di-S-C₅(5) causes an artifactual increase in fluorescence in the presence or absence of neutrophils. 4 Zymosan was opsonized with fresh serum after extraction and washing.

Cytochrome-b was identified in reduced-minus-oxidized difference spectra of granulocytes reduced with sodium dithionite. 25 The cell suspension containing 2 × 10⁶ PMN in 2 ml balanced salt solution with 1% dextran was placed in the sample and reference cuvettes of a Perkin-Elmer model 557 double-beam spectrophotom-
eter. Light scattering and oxidized spectra from 400 to 600 nm were recorded. Then, a few grains of sodium dithionite were added and further difference spectra were recorded. The quantity of cytochrome-b was calculated from the peak heights of duplicate spectra (within 10% of each other) using the absorption coefficient of human neutrophil plasma membrane cytochrome-b$_{553}$ [\(\Delta\epsilon \, mM^{-1} \cdot cm^{-1}\)]$^{14}$.

**RESULTS**

**Bacterial Killing**

The rate of killing of *S. aureus* by the patient’s intact PMN was significantly less when compared to control cells (Fig. 1). After 90 min of incubation, the mean number of ingested organisms that had been killed was within 2 standard deviations of the number killed by control cells. Uniform distribution of dead organisms was noted in the patient’s cells and in those of her mother and sister, whose cells killed *S. aureus* at control rates, suggesting that the behavior of the cell population was relatively homogeneous.

**Respiratory Burst in Intact Cells**

Quantitative NBT reduction by the patient’s PMN occurred at 3% of that by PMN from family members and unrelated controls (Table 1). Over 85% of the patient’s cells were faintly positive on NBT slide test, with both PMA stimulation and opsonized zymosan ingestion; there were no strongly positive cells and 12%–15% negative cells. Over 90% of her mother’s PMN were strongly positive, and 7% weakly positive, with no negative cells. The father and sister’s cells were greater than 95% strongly positive.

The initial rate of O$_2^-$ production by the patient’s neutrophils was less than 2% of that by control PMN exposed to soluble and particulate stimuli. Rates of O$_2^-$ production by the patient’s mother’s (Table 1) and father’s and sister’s (data not shown) PMN were comparable to that by control PMN. The initial rate of O$_2^-$ production by the patient’s monocytes was similar to that by her PMN. Monocytes from her mother and unrelated controls produced O$_2^-$ at approximately 63% of the rate of PMN.

![Graph showing killing of S. aureus by neutrophils](image)

**Fig. 1.** Killing of *S. aureus* by neutrophils (mean ± 1 standard deviation). (□) Patient; (○) control. Percentage of total intracellular *S. aureus* that was killed by neutrophils was assessed by differential fluorescent staining with acridine orange. Live organisms stain green, dead organisms stain red. Results are expressed as the percentage of dead (red-staining) organisms of the total intracellular organisms (red plus green).

| Table 1. Rates of NBT Reduction and Superoxide Production by Intact PMN and Monocytes |
|----------------------------------|---------|---------|-------|
| Patient                          | Mother  | Control |
| Quantitative NBT reduction (ng formazan/min/10$^5$ PMN) | 6 ± 1 | 196 ± 6 | 200 ± 8 |
| Initial rate O$_2^-$ production by PMN (nmole O$_2^-$ /10$^5$ PMN) | | |
| PMA                              | 0.1     | 7.0     | 7.7   |
| Oposazoned zymosan               | 0.4     | 9.7     | 9.2   |
| Initial rate O$_2^-$ production by monocytes (nmole O$_2^-$ /min/10$^6$ monocytes) | | |
| PMA                              | 0.1     | 4.6     | 5.0   |
| Oposazoned zymosan               | 0.2     | 6.1     | 5.8   |
| Lag time O$_2^-$ production by PMN (sec) | | |
| PMA                              | 60      | 60      | 67    |
| Oposazoned zymosan               | 32      | 29      | 33    |
| Lag time O$_2^-$ production by monocytes (sec) | | |
| PMA                              | 62      | 61      | 65    |
| Oposazoned zymosan               | 33      | 28      | 30    |
The lag time, an index of the time necessary for activation of the O$_2^-$ generating system, was the same for both the patient's and control PMN and monocytes. Both the patient's cells and control cells consistently displayed a shorter lag time after zymosan stimulation (28–33 sec) than PMA stimulation (60–67 sec).

**Kinetics of Superoxide Production**

The rates of NADPH-dependent superoxide production by podosomes and the particulate fraction were comparable, but there was greater variability in activity among preparations of podosomes than the particulate fraction, especially at low substrate concentrations. Superoxide production by preparations from controls and family members was maximal at 0.1 mM NADPH, but barely detectable in material from the patient's PMN. At 10 mM NADPH, the rate of O$_2^-$ production was similar in patient and control material. Figure 2 shows a Lineweaver-Burk analysis of the kinetic data obtained with the particulate fraction, demonstrating a lower apparent affinity (higher $K_{m,app}$) for NADPH in the patient's particulate fraction compared to control, and similar maximum velocities. Table 2 shows that the $K_{m,app}$ for NADPH in the patient's particulate fraction was 3–4 times that in control material, and the $V_{max}$ was 85% of control.

The kinetics of NADH-dependent superoxide production by patient and control particulate fraction were similar (Fig. 3). The $K_{m,app}$ could not be precisely determined because of nonlinearity at low NADH concentrations.

**Duration of Superoxide Production**

Superoxide production by PMA-stimulated normal PMN continued at a linear rate for 8–10 min and then gradually plateaued. Addition of 50 n mole cytochrome-c to sample and reference cuvettes, or 10 $\mu$g SOD to the reference cuvettes, failed to restore the rate of O$_2^-$ production, indicating that neither exhaustion of substrate nor destruction of SOD accounted for the lowered rate of reaction. By 30 min, superoxide production had virtually ceased. A similar time course of fall in the rate of superoxide production was seen in both podosomes and the particulate fraction.

In contrast, superoxide production by the patient's intact PMN continued at the initial rate for 28–32 min and did not cease until 70–90 min of incubation. The precise time varied with experiments. Superoxide production by the particulate fraction also continued for 25–30 min at 0.1 mM NADPH, but began to decline by 15 min of incubation at 1.0 mM NADPH.

**Depolarization**

Changes in fluorescence, indicating the occurrence of membrane depolarization, were seen with both fluorescent dyes and with exposure of cells to particulate (opsonized zymosan) and soluble (PMA and...
concanavalin-A) stimuli (Fig. 4). Depolarization measured with di-S-C$_3$(5) is seen as an increase in fluorescence (Fig. 4, A and B), followed by decreased fluorescence or "apparent repolarization," which occurs because of oxidation of the dye by products of oxygen and myeloperoxidase. Depolarization of the patient's cells occurred later and was of lower magnitude than depolarization of control cells. In both instances, depolarization preceded measurable O$_2^-$ production. The late decrease in fluorescence was markedly slower than that seen with control cells.

Depolarization measured with di-O-C$_5$(3) is seen as a decrease in fluorescence. This dye is not affected by products of the respiratory burst, and return to baseline is not seen. Again, both the extent of depolarization and the temporal pattern were different from simultaneous controls stimulated with concanavalin-A (Fig. 4C).

**Cytochrome-b**

Reduced-minus-oxidized difference spectra, upon reduction of intact granulocytes with dithionite, demonstrated identical patterns in patient and control cells. The patient's intact PMN contained 110 pmole cytochrome-b/mg cell protein, compared to 105

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**Fig. 3.** NADH-dependent production of superoxide by particulate fraction of neutrophils. The initial rate of O$_2^-$ production by material from the patient (open circles) and a normal control (closed circles) is plotted against the concentration of NADH in the reaction mixture.

**Fig. 4.** Membrane depolarization with activation of PMN. PMA were equilibrated with fluorescent dyes prior to addition of stimuli, indicated by arrows. All changes in fluorescence are given on the same scale of arbitrary units. Dotted line: control cells. Solid line: patient cells. (A) Change in fluorescence of $2 \times 10^{-5} \text{ M} \text{ di-S-C}_3$(5) in response to 1 $\mu\text{g}$ PMA/ml. (B) Change in fluorescence of $2 \times 10^{-5} \text{ M} \text{ di-S-C}_3$(5) in response to 3 $\mu\text{g}$ opsonized zymosan/ml. (C) Changes in fluorescence of $5 \times 10^{-5} \text{ M} \text{ di-O-C}_5$(3) in response to 100 $\mu\text{g}$/ml concanavalin-A/ml, in the presence of 5 $\mu\text{g}$ cytochalasin-B/ml.
pmole/mg protein in control cells, similar to published norms.25

Other Studies

Absolute neutrophil counts were consistently normal or elevated. Myeloperoxidase stains were positive. Serum immunoglobulins, including IgE, were all slightly elevated. Lymphocyte numbers and subpopulations were normal. Neutrophil and monocyte chemotaxis were normal in Boyden chambers and Rebecub skin windows. Ingestion of S. aureus, Candida albicans, and of endotoxin-coated oil droplets opsonized with complement was normal. G6PD and glutathione peroxidase activity were normal in erythrocytes and neutrophils. The rate of oxidation of 14C-1-glucose to 14CO2 in response to exogenous production of hydrogen peroxide by leucine and l-amino acid oxidase was comparable to control values.

DISCUSSION

This child has an apparently autosomal recessive disorder that has several distinctive features. She has been generally well for her entire life, experiencing only mild pulmonary aspergillosis, which has resolved without specific therapy. She would not have been evaluated on the basis of her own clinical manifestations, but was found to have abnormal neutrophil function because of her brother’s fatal illness. Her mother, father, and sister have normal neutrophil superoxide production and NBT reduction, and no clinical abnormalities suggestive of the carrier state.

Activation of the membrane-associated oxidase was normal in the patient’s PMN and monocytes, as judged by the normal lag times. Cells depolarized in response to both soluble and particulate stimuli, but there were quantitative and qualitative differences in the pattern of fluorescent changes in the patient’s cells compared to normal. This finding was distinct from the abnormalities in the X-linked and autosomal recessive forms of CGD in which no O2- is produced and no membrane depolarization is seen.3

Both the rate of NBT reduction and the initial rate of superoxide production by the patient’s PMN and monocytes were approximately 3% of the rates by control cells. Despite this major difference in intact cells, kinetic data on membrane preparations revealed 3–4-fold difference in the K_{m}^{app} of the enzyme. Similar rates of NBT reduction and superoxide production (3% of control values) were found in PMN from two other unrelated male patients with X-linked disorders, both of whom had low-affinity NADPH-dependent oxidases and relatively mild clinical courses.7,25 However, the K_{m}^{app} of the oxidase was 50–60 times normal in one patient’s PMN,25 compared to 3–4 times normal in our patient. The fact that the comparatively minimal alteration in enzyme affinity is associated with levels of whole cell superoxide production comparable to that seen with a much lower affinity enzyme suggests that the availability of NADPH in intact cells becomes rate-limiting with even minor alterations in enzyme kinetics. The absence of the respiratory burst in severe G6PD deficiency, in which NADPH is rapidly depleted and not regenerated, further supports this concept.6 All three reported cases with different changes in NADPH-affinity have normal affinity for NADH but abnormal O2- production, supporting other evidence that NADPH is the true intracellular substrate for the enzyme.

Most cases of chronic granulomatous disease are X-linked and associated with apparently defective activation of the membrane-associated oxidase. In the absence of activation of the oxidase, no information can be obtained about its kinetic properties. Thus, the X-linked nature of most cases of CGD does not imply that the oxidase itself arises from genetic information carried on the X-chromosome. The description of two kinetically abnormal oxidases inherited as X-linked traits does suggest that some determinant of enzyme activity is carried on the X-chromosome. One of these patients25 has low levels of cytochrome-b, which is present in both the plasma membrane and secondary granule membranes of PMN.37 The associated low cytochrome-b levels and high K_{m}^{app} for NADPH suggest that cytochrome-b may have a structural role in the O2- generating system of phagocytes. The patient described in this article has an apparently autosomal recessive disorder associated with abnormal enzyme kinetics despite normal enzyme activation and normal cytochrome-b. This finding would be compatible with the hypothesis that mutations on the X-chromosome and autosomes could both result in low-affinity oxidases. The NADPH-dependent O2- generating system requires interaction between protein and lipid.28 Thus, the finding of decreased substrate affinity may not necessarily imply an alteration in the active site of the protein itself. Allosteric phenomena and changes in the lipid milieu may also alter the enzyme’s affinity for its substrate. Our observation of both quantitative and qualitative abnormalities in depolarization suggests the possibility of a defect in a component of the enzyme system apart from the active site.

Killing of S. aureus was considerably delayed, but after 90 min, most of the ingested organisms had been killed. This observation is compatible with the fact that superoxide production continues over a much longer period of time than occurs in normal PMN. The delayed killing of S. aureus seen in this patient’s cells is
similar to the defect observed in patients with myeloperoxidase deficiency. The clinical course of this patient is also similar to MPO-deficiency in the relatively minor nature of infections experienced by affected persons. Although the rate of O$_2^-$ production by the patient’s cells is low, the total amount produced over 60–90 min appears to be sufficient to kill catalase-positive intracellular organisms. Killing of fungi by monocytes may be similarly delayed and may be less efficient because of the lower rate of O$_2^-$ production by monocytes compared to PMN. The long time course of superoxide production when the rate is low supports suggestions by others that products of the respiratory burst are involved in its inactivation.$^{29}$ Myeloperoxidase is present in the particulate fraction and may participate in inactivation of the oxidase in our system. The prolonged respiratory burst seen with decreased rates of superoxide production in this patient and in one previously reported$^7$ represent the converse of the abbreviated respiratory burst due to early toxicity of H$_2$O$_2$, which accompanies glutathione reductase deficiency.$^{30}$

The biochemical and physiologic abnormalities described in this variant of CGD have added to our understanding of the relationship between the kinetics of O$_2^-$ production and the bactericidal capacity of the granulocyte. In addition, just as activation defects can occur in both X-linked and autosomal recessive forms of CGD, abnormalities in the affinity of the enzyme for its substrate have now been shown to have both inheritance patterns.

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