Use of the Integrated Steady State Rate Equation to Investigate Product Inhibition of Human Red Cell Adenosine Deaminase and its Relevance to Immune Dysfunction*

(Received for publication, November 4, 1977)

WILLIAM R. A. OSBORNE, SHI-HAN CHEN, AND C. RONALD SCOTT

From the Department of Pediatrics, University of Washington, School of Medicine, Seattle, Washington 98195

SUMMARY

The analysis of progress curves using the integrated rate equation was applied to the adenosine deaminase-catalyzed conversion of adenosine to inosine.

Adenosine deaminase was purified from human red blood cells of the ADA 1, ADA 2, and ADA 2-1 types. For all three types, no measurable product inhibition by inosine was observed.

These results do not confirm the hypothesis that inosine accumulation in purine nucleoside phosphorylase deficiency causes adenosine deaminase inhibition, resulting in a common mechanism for the immune defects related to these two enzyme deficiencies.

Human red cell adenosine deaminase (adenosine aminohydrolase EC 3.5.4.4) catalyzes the conversion of adenosine to inosine and ammonia and is a monomeric protein of about 30,000 molecular weight (1, 2). The commonly inherited forms are controlled by two allelic genes designated ADA 1 and ADA 2 (3). In the system of purine rearrangement, the inosine produced by the adenosine deaminase reaction is converted to hypoxanthine and ribose 1-phosphate by the enzyme purine nucleoside phosphorylase (4).

In humans, a genetic deficiency of adenosine deaminase causes a failure of normal T and B lymphocyte function and leads to early death of children from bacterial or viral infection (5, 6). A genetic deficiency of purine nucleoside phosphorylase also causes immune dysfunction, but it is limited to T cell function (7, 8). It has been proposed that the accumulation of inosine that results from purine nucleoside phosphorylase deficiency inhibits adenosine deaminase, the preceding enzyme in the purine reutilization cycle, and thus a common mechanism is responsible for both immune defects (9-11).

The usual kinetic analysis to estimate product inhibition involves the measurement of initial rates of reaction at various substrate concentrations with and without added inhibitor (12). However, when using the direct spectrophotometric assay for adenosine deaminase, this approach is impractical because at 265 nm, the wavelength of maximum absorbance difference between substrate and product, both have high extinction coefficients. Thus, at the concentrations of inosine required to estimate any product inhibition, absorbance values are encountered that preclude reliable assays. The analysis of progress curves using the integrated rate equation (13, 14) is not subject to this limitation and therefore was used to test for any product inhibition of the adenosine deaminase reaction.

MATERIALS AND METHODS

Purification of Adenosine Deaminase - Outdated donor blood was used as the source of human red cells. Hemolysates were prepared and the stroma was removed as described by Brow nson and Spencer (15). Starch gel electrophoresis was by the method of Spencer et al. (3). Hemoglobin-free hemolysates were prepared using dry CM-C-50 Sephadex (1), and then were subjected to affinity chromatography (16) to obtain purified adenosine deaminase. Red cells used were of phenotypes ADA 1, ADA 2, and ADA 2-1, and each type of enzyme was purified separately. All preparations were free of purine nucleoside phosphorylase activity.

Enzyme Assays - Adenosine deaminase and purine nucleoside phosphorylase were assayed spectrophotometrically by the methods of Kalckar (17). A radioisotope assay system for adenosine deaminase using [14C]adenosine was also employed (18). Heat Denaturation - The first order rate constants for heat denaturation of a purified ADA 1 preparation, with and without the addition of various amounts of human serum albumin, were performed at 54° in 0.1 M phosphate buffer, pH 7.0. The rate constants were determined by plotting the slope of the temperature versus time relationship.

Progress Curves - Experiments using the three purified adenosine deaminase phenotypes were conducted with initial substrate concentrations of 60, 90, and 120 μM in 0.1 M phosphate buffer, pH 7.0, and human serum albumin at a final concentration of 0.1 mg/ml. The reaction progress curves were monitored at 265 nm with a Gilford recording spectrophotometer equipped with a recorder and a circulating water bath to maintain a temperature of 30°. The curves were recorded until the reaction had finished, a period of 1 to 4 h depending upon substrate/enzyme concentrations.

To test for any inactivation of adenosine deaminase during the assay, the procedure of Selwyn (19) was used. For this, a constant initial substrate concentration of 90 μM in 0.1 M phosphate buffer, pH 7.0, and three different enzyme concentrations of an ADA 1 preparation were used. These reactions were carried out with and without the addition of human serum albumin at a final concentration of 0.1 mg/ml. The reactions were monitored by recording the absorbance at 265 nm and 30°.

RESULTS AND DISCUSSION

The results of the heat denaturation experiments are shown in Fig. 1. The purified adenosine deaminase preparation was rapidly denatured, but with the serial addition of human serum albumin, it became increasingly more stable until a limit was reached at a k value of about 1.7 × 10^-4 s^-1, corresponding to a total protein concentration of 0.1 mg/ml. In contrast to this stabilizing effect of added protein, a decrease in heat stability of adenosine deaminase with increasing ionic strength of phosphate buffer at pH 7 has been reported (1). The addition of inorganic ions most likely causes a loosening of the protein structure by disrupting the ionic forces between the amino acid residues of the outer (surface) protein strands, whereas increasing protein concentration...
probably stabilizes by promoting association and thus protection of these forces.

The results of the Selwyn procedure (19) to test for any enzyme inactivation are recorded in Fig. 2 as inosine formation versus the multiplication of enzyme concentration by the reaction time. The reaction curves involving the three enzyme concentrations with the addition of 0.1 mg/ml of human serum albumin lie on the same line, indicating that there is no time-dependent adenosine deaminase inactivation. However, the reactions carried out without the addition of human serum albumin do not co-plot, showing a time-dependent process, i.e. heat inactivation of the enzyme. These results are in accord with the heat denaturation experiments shown in Fig. 1 and validate the use of the integrated rate equation for reactions carried out in the presence of added albumin protein.

The plots of progress curves using an ADA 1 preparation are shown in Fig. 3. The presentation used is that derived from the integrated rate equation of Foster and Niemann (13). The three substrate levels of 60, 90, and 120 μM gave slopes of 27.1 ± 1.1, 26.8 ± 0.9, 27.2 ± 1.1 μM and ordinate intercepts of 3.59, 3.60, and 3.61, respectively. By analysis of covariance, these lines are not significantly different at α = 0.05. Therefore, there is no product inhibition by inosine of the adenosine deaminase reaction, and the value of the slopes is the Michaelis constant $K_m$. When the combined data were analyzed, a slope value of $K_m = 27.0 ± 0.6$ μM was determined. Repeating the progress curves with ADA 2-1 and ADA 2 preparations gave $K_m$ values of 26.1 ± 1.1 and 27.0 ± 0.8 μM, respectively.

Furthermore, from the integrated rate equation, the value of the slope is $-K_p/[K_p + S_o]/(K_p - K_m)$, where $K_p = product~inhibition~constant$, $S_o = initial~substrate~concentration$, and $K_m = Michaelis~constant$. Thus, for the slopes to be common the limit $K_p → ∞$, $(K_p + S_o)/(K_p - K_m) = 1$. By substituting in this equation $K_p = 27 μM$ and $S_o = 100 μM$, it is evident that at $K_p = 2$ mm, the experimental slope values would not be significantly different. Accepting from this a minimum $K_p$ value of 2 mm, there can be little inhibition of adenosine deaminase in patients deficient in purine nucleoside phosphorylase, as their highest recorded levels of inosine in plasma have not been greater than 0.2 mM (8, 20).

Radioisotope assays were performed with an ADA 1 prepa-

Several values have been reported for the competitive product inhibition of human red cell adenosine deaminase. For example, $K_p = 116 μM$ (30°), Agarwal et al. (2); $K_p = 600 μM$ (37°), Van der Weyden and Kelly (21); $K_p = 700 μM$ (37°) Daddona and Kelly (22). Because a statistical analysis of the kinetic data was not given in any of these reports, it is difficult to estimate the significance of these values.

From these experiments, it can be concluded that there is no definable product inhibition of the adenosine deaminase reaction. Thus, the hypothesis is not tenable that in purine nucleoside phosphorylase deficiency, inosine accumulation causes inhibition of adenosine deaminase, resulting in a

1 U. Ochs, unpublished observations.
common mechanism for the immune defects associated with these two enzyme deficiencies.

REFERENCES

Use of the integrated steady state rate equation to investigate product inhibition of human red cell adenosine deaminase and its relevance to immune dysfunction.
W R Osborne, S H Chen and C R Scott


Access the most updated version of this article at [http://www.jbc.org/content/253/2/323](http://www.jbc.org/content/253/2/323)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/253/2/323.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/253/2/323.full.html#ref-list-1](http://www.jbc.org/content/253/2/323.full.html#ref-list-1)