Partial Purification and Properties of the Common Inherited Forms of Adenosine Deaminase from Human Erythrocytes

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1. The partial purification of adenosine deaminase, types 1, 2 and 2–1, from human erythrocytes is described. 2. The isoenzyme components characteristic of the three forms of the enzyme were partially resolved by chromatography on DEAE-Sephadex. 3. Gel chromatography of the various forms of the enzyme gave estimates of the molecular weights in the range 30000–35000. 4. Electrophoresis in starch gels containing increasing percentages of starch did not reveal any differences in molecular weight between the genetic variants or their isoenzyme components. 5. Analytical isoelectric-focusing experiments in polyacrylamide gels gave the following pI values for the four isoenzyme components present in type 2–1 erythrocytes: 4.70, 4.83, 4.94 and 5.06. 6. All forms of the enzyme gave K_m values for adenosine of about $30\mu\text{M}$ and K_i values of about $8\mu\text{M}$ for the competitive inhibitor purine riboside. 7. Reaction rates of the type 1 and 2 enzymes were measured at different temperatures. Both enzymes gave values for the energy of activation for hydrolysis of adenosine of about 33.4 kJ/mol (8 kcal/mol). 8. Heat inactivation of all forms of the enzyme was markedly dependent on ionic strength, the rate of inactivation increasing with increasing ionic strength. The type 1 and type 2 forms of the enzyme differed significantly in their susceptibility to heat inactivation. From the variation of rates of inactivation with temperature, values were obtained for the energies of activation for the heat inactivation of both enzymes as follows: type 1 enzyme 275.5 kJ/mol (65.9 kcal/mol) and type 2 enzyme 241.6 kJ/mol (57.8 kcal/mol.).

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) has been shown to exist in different inherited forms in erythrocytes of different individuals (Spencer *et al.*, 1968). The different forms of the enzyme are distinguishable by their electrophoretic mobilities in starch-gel electrophoresis. Studies of families support the hypothesis that the commonly observed forms are controlled by two allelic genes, designated 1 and 2. The three types of individual expected on this basis have been observed: type 1 and type 2 individuals, whose erythrocytes contain respectively the type 1 and type 2 forms of the enzyme, and type 2–1 individuals, whose erythrocytes contain both forms of the enzyme.

When haemolysates, prepared from erythrocytes of all types, are analysed by starch-gel electrophoresis in conjunction with a specific stain for adenosine deaminase activity, a pattern of at least three isoenzymes is observed. The relative proportions of the isoenzymic forms alter with increasing age of the haemolysate (Spencer *et al.*, 1968) in a manner which suggests that some of the multiple forms observed

probably arise as a result of secondary modifications of the original gene product (Harris, 1970).

In the present work, the enzyme has been extensively purified from human erythrocytes and the various isoenzyme components have been separated. Preliminary studies on some kinetic and heat-stability properties are reported.

Experimental

Materials

Chemicals. Adenosine, nucleoside phosphorylase and xanthine oxidase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Hydrolysed starch was obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada. CM-Sephadex (C-50) and DEAE-Sephadex (A-50) were obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. Phenazine methosulphate was a product of Ralph N. Emanuel Ltd., Wembley, Middx., U.K., and M.T.T. tetrazolium salt was from B.D.H. Ltd., Poole, Dorset, U.K. Ampholytes were supplied by L.K.B. Instruments Ltd., South Croydon, Surrey, U.K.

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Buffers. All pH measurements were done at room temperature. Anionic buffers were prepared by adding NaOH to the appropriate acid or acid salt to give the required pH and molarity of anion. Similarly, cationic buffers were prepared by using the free base and HCl.

Methods

Starch-gel electrophoresis. Starch gels (12%, w/v) were prepared in 5mm-sodium succinate buffer, pH 6.0, and 100mm-succinate buffer of the same pH was used in the electrode compartments. Gels were poured to a depth of 0.5cm into glass moulds (16cm × 28cm). Horizontal electrophoresis was done in a cold-room with an applied voltage of 3V/cm for 16h. The gels were then sliced and stained as described by Spencer *et al.* (1968).

Analytical isoelectric focusing. The pI values of the isoenzyme components of types 1 and 2-1 preparations were determined by isoelectric focusing in 5% (w/v) polyacrylamide gels containing L.K.B. ampholytes. The gels were prepared in a mould (0.1cm × 16cm × 20cm) as described by Vesterberg (1972). Focusing was carried out for 24h and the current was controlled to give a maximum power of 3W. Enzyme activity was located with an agar overlay as described by Spencer *et al.* (1968).

Determination of molecular weight. Approximate molecular weights were determined by gel filtration through columns of Sephadex G-75 (Andrews, 1965). Gel equilibrated in 10mm-phosphate buffer, pH 7.0, was packed into a column (2.5cm × 40cm). The following proteins were used as standards: cytochrome *c* (mol.wt. 12400); horse myoglobin (mol.wt. 17800); bovine serum albumin (mol.wt. 68000); chymotrypsinogen (mol.wt. 25000).

The starch-gel-electrophoresis method of Smithies (1962) was used to investigate possible differences in molecular weight between the various isoenzyme components. A gel containing 14% (w/v) starch, prepared as described above, was poured into glass moulds to give a strip (2cm × 28cm). When this strip had set, additional strips containing 16, 18 and 20% (w/v) starch were made in turn, so that in the final composite gel the strips were in lateral contact. The gel was then run and sliced as described above.

Adenosine deaminase activity. This was measured by using essentially the method of Kalckar (1947). Assays were carried out at 30°C in a Unicam SP.800 spectrophotometer with silica cuvettes of 1cm light-path. The standard reaction mixture contained 80 μM-adenosine in 100mm-phosphate buffer, pH 7.0, in a final volume of 3ml. Reaction rate as measured by the decrease in E_{265} was proportional to enzyme concentration provided that the extinction change did not exceed 0.03/min. Protein concentration was measured as described by Brownson & Spencer (1972). Specific activity is expressed as the number of μmol of adeno-

sine converted/min per mg of protein under the conditions described. Activity of column eluates is given as the change in E_{265} /min per ml of eluate.

Partial purification of adenosine deaminase from human erythrocytes. As the enzyme was very stable it was convenient to carry out all the purification procedures at room temperature. The erythrocytes used in these experiments were from individual donors whose enzyme type was confirmed on repeat samples. Whole blood was obtained from the sources quoted by Brownson & Spencer (1972). Crude haemolysates were prepared and the stroma was removed as described by Brownson & Spencer (1972); all solutions in the purification procedure contained 1mm-dithiothreitol. The supernatant solution remaining after removal of stroma was used in further purification steps.

After several preliminary experiments the following simplified procedure was adopted for removing haemoglobin. Crude haemolysate representing 200ml of packed erythrocytes was adjusted to pH 5.8 with 0.1M-HCl. Dry CM-Sephadex (6g) was added slowly with stirring to the haemolysate. The mixture was stirred for 30min and the pH adjusted to 5.8 if necessary. The resin was filtered off by using a Buchner pump and a further 4g of dry resin was added to the filtrate. The mixture was stirred for a further 30min and then the second batch of resin was removed, leaving a filtrate essentially free of haemoglobin. At this stage the product could be either freeze-dried and stored, or concentrated by pressure dialysis by using an Amicon Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) with a UM-10 filter. The enzyme was purified further by chromatography on a column (2.5cm × 40cm) of DEAE-Sephadex equilibrated against 45mm-imidazole-HCl buffer, pH 6.0. Enzyme concentrate (10-20ml) was dialysed overnight against 50mm-imidazole-HCl buffer, pH 6.0, and was then applied to the column. The column was eluted with a linear gradient of NaCl as follows: the mixing vessel contained 500ml of 50mm-imidazole-HCl buffer, pH 6.0, and the reservoir vessel of identical dimensions contained 500ml of 0.2M-NaCl in the same buffer. A flow rate of 30-40ml/h was used and fractions (6ml) were collected and analysed for adenosine deaminase activity and protein. The Cl⁻ concentration was estimated on every twentieth fraction by titration with AgNO₃. Fractions containing enzyme activity were pooled and concentrated by pressure dialysis.

Results

Purification and separation of isoenzymes

Several procedures including preparative electrophoresis and isotachopheresis in polyacrylamide gels and stepwise elution from DEAE-Sephadex were

Fig. 1. Chromatography of a crude haemolysate of type 2-1 erythrocytes on DEAE-Sephadex

Experimental conditions are described in the text. ○, Adenosine deaminase activity ($\Delta E_{265}/\text{min per ml}$); ●, protein (E_{280}); ▲, [NaCl]. For further details of peaks 1, 2, 3 and 4 see Plate 1 and the text.

investigated. However, the purification and resolution achieved by gradient elution on DEAE-Sephadex was far superior to that obtained with any of the above methods. By varying the column length, steepness of Cl^- gradient and flow rate through the column partial resolution of isoenzyme forms was possible. Short (40cm) columns gave better resolution than 100cm columns. Cl^- gradients from 0–0.15M up to 0–1M were tested, and a gradient of 0–0.2M was finally adopted. Flow rate through the column was particularly critical. Slow rates (10–20ml/h) gave no resolution of the isoenzymes; optimum results were obtained with a flow of 30–40ml/h through a 40cm column. Fig. 1 shows the resolution achieved with an enzyme preparation from type 2-1 erythrocytes. Similar separations were obtained with preparations from types 1 and 2 erythrocytes. Individual fractions representing the peaks of enzymic activity were concentrated by the addition of dry Sephadex G-25 and analysed by starch-gel electrophoresis. Except for the fraction corresponding to the fourth peak of enzyme activity the fractions analysed appeared to contain individual isoenzyme components (Plate 1). However, the amount of such material available precluded the possibility of a more rigorous purification or of a detailed study of the properties of individual isoenzymes. The experiments that follow, therefore, were carried out on pooled fractions of enzyme activity collected from columns of DEAE-Sephadex. A typical purification scheme is given in Table 1.

Analytical isoelectric focusing

In these experiments crude haemolysates and partially purified preparations from erythrocytes of

types 1 and 2-1 individuals were examined by using ampholyte mixtures which covered the pI ranges 3–6 and 4–6. Preparations from several individual donors of both types gave pI values for the various isoenzyme components that agreed closely with each other. Typical pI values for the components of a type 1 enzyme preparation were 4.70, 4.83 and 4.93, which correspond to the components observed in starch gels at pH 6.0 in order of decreasing anodic mobility. The corresponding pI values for a type 2-1 enzyme preparation were 4.70, 4.83, 4.94 and 5.06.

Molecular weights

Molecular weight as determined by gel chromatography of both crude haemolysates and purified preparations from types 1, 2 and 2-1 erythrocytes was in the range of 30000–35000. Recent reports (Ma & Fisher, 1968; Akedo *et al.*, 1972) suggest that adenosine deaminase from a variety of sources exists in both high- and low-molecular-weight forms. The possible existence of a monomer–polymer relationship between the isoenzyme forms of the enzyme from human erythrocytes was investigated in crude haemolysates by the method of Smithies (1962). Table 2 shows the mobilities of the four isoenzyme bands observed in type 2-1 haemolysates in each of the starch concentrations that was used. The relative mobilities, taking the fastest moving isoenzyme (*a*), as 1, are shown in parentheses. Since the relative mobilities are constant in the four concentrations of starch this appears to rule out the possible existence of significant molecular weight differences between the isoenzymes.

Table 1. *Purification of adenosine deaminase from type 1 erythrocytes*

Fraction	Vol. (ml)	For details see the text.		Specific activity (units/mg of protein)	Purification factor	Yield (%)
		Total activity (units)	Total protein (mg)			
Crude haemolysate	300	22.0	37000	0.00059	1	100
Supernatant liquid remaining after batchwise CM- Sephadex procedure	220	19.0	1120	0.017	29	87
Supernatant liquid concen- trated by pressure dialysis	20	18.0	1040	0.017	29	82
Fractions containing adeno- sine deaminase activity from DEAE-Sephadex column concentrated by pressure dialysis	10	10.5	2	5.3	8500	48

Table 2. *Effect of starch concentration on the electrophoretic mobilities of the isoenzyme components of adenosine deaminase, type 2-1*

Mobilities are expressed as distance travelled under the conditions described in the Experimental section. Values shown in parentheses are relative to the mobility of the component (*a*) which moves fastest towards the anode. Isoenzymes are designated *a*, *b*, *c* and *d* in order of decreasing anodal mobility.

Isoenzyme	Starch concn. (%) ...	Mobility (cm)			
		14	16	18	20
<i>a</i>		6.5 (1)	5.80 (1)	4.95 (1)	4.20 (1)
<i>b</i>		5.6 (0.86)	4.95 (0.85)	4.20 (0.85)	3.70 (0.88)
<i>c</i>		4.4 (0.68)	3.85 (0.66)	3.35 (0.68)	2.80 (0.67)
<i>d</i>		3.1 (0.48)	2.70 (0.47)	2.25 (0.46)	1.95 (0.46)

Table 3. *Michaelis constants for adenosine and inhibitor constants for purine riboside for the different genetic forms of adenosine deaminase from human erythrocytes*

The constants, $\pm$ S.E.M. (for six experiments), were calculated from initial-velocity (v_0) values measured as described in the text.

Enzyme type	K_m for adenosine (μ M)	K_i for purine riboside (μ M)
1	30.8 ± 2.9	8.4 ± 0.73
2	28.0 ± 2.2	8.7 ± 0.67
2-1	29.0 ± 2.7	8.7 ± 0.65

Kinetic properties

Initial-velocity (v_0) studies were carried out at 30°C on partially purified preparations. Velocities were determined in triplicate at several concentrations of

substrate and the results were analysed by computer by using the program devised by Wilkinson (1961). Preparations from types 1, 2 and 2-1 erythrocytes all gave K_m values for adenosine of 28–30.8 μ M (Table 3).

Adenosine deaminase from many sources is competitively inhibited by purine ribonucleoside. This inhibitor was tested against the erythrocyte enzymes and the initial-velocity results analysed by Lineweaver–Burk and Dixon plots gave K_i values of 7.8–8.7 μ M for the various preparations (Table 3).

Effect of temperature on reaction rate

In a simple one-substrate reaction such as that catalysed by adenosine deaminase the energy of activation for the reaction may be determined by measuring V_{max} at several temperatures on the assumption that only one enzyme–substrate complex is involved in the catalytic mechanism. Shortage of material in the case of the rare type 2 variant precluded determination of V_{max} by initial-velocity

EXPLANATION OF PLATE I

Starch-gel electrophoresis of fractions resolved by chromatography on DEAE-Sephadex

Sample 0 corresponds to the original type 2-1 haemolysate. Samples 1, 2, 3 and 4 are concentrates prepared from the correspondingly labelled peak fractions shown in Fig. 1.

Table 4. *Effect of buffer concentration on the first-order rate constants for the thermal inactivation of adenosine deaminase, type 1*

Rates of inactivation were measured at 57.5°C in phosphate buffer, pH7.

Concn. of buffer (mM)	0	5	10	50	100-200
Rate constant (min ⁻¹)	0.0021	0.0120	0.0129	0.0226	0.0286

Fig. 2. *Heat inactivation of partially purified adenosine deaminase types 1 (●), 2 (○) and 2-1 (▲) at 56°C in 100mM-phosphate buffer, pH7*
For details see the text.

measurements at various concentrations of substrate and at different temperatures. The alternative approach of measuring V_{max} directly by using saturating concentrations of adenosine was not possible with the assay method employed, since the high extinction values were outside the range of the Unicam SP.800. The maximum starting concentration of adenosine that could be used was 80 μ M, i.e. only three to four times the K_m value for this substrate. It was decided to compare the value for the activation energy, obtained from V_{max} values by using reciprocal plots with that derived by direct measurement of initial velocity (v_0) in 80 μ M-adenosine; for this purpose a purified preparation of the type 1 enzyme, which was readily available, was used. The energies of activation from Arrhenius plots were 39.7 and 36.4 kJ/mol (9.5 and 8.7 kcal/mol) from the V_{max} and v_0 values respectively. As these values agree within experimental error, it seemed justifiable to compare the activation energies of the type 1 and type 2 enzymes by using values derived from v_0 measurements in 80 μ M-adenosine. These measure-

Fig. 3. *Effect of temperature on the first-order rate constants for the heat inactivation of partially purified adenosine deaminase types 1 (●) and 2 (○) in 100mM-phosphate buffer, pH7*
For details see the text.

ments were made in 100mM-phosphate buffer, pH7.0, at 10, 20, 30 and 40°C on purified enzymes from types 1, 2 and 2-1 erythrocytes. All the Arrhenius plots were linear and gave values of 36.4, 32.6 and 35.5 kJ/mol (8.7, 7.8 and 8.5 kcal/mol) for the activation energies of the types 1, 2 and 2-1 enzymes respectively. The results show no significant difference for this parameter between the genetic forms of the enzyme.

Heat-inactivation studies

In these experiments purified enzyme preparations were incubated at a particular temperature and samples were removed at suitable intervals and assayed in the standard assay procedure. Preliminary experiments showed that the rate of inactivation at 57.5°C was strongly dependent on the concentration of phosphate buffer (pH7.0) in the incubation medium. Table 4 shows the effect of buffer concentration on the first-order rate constant for the heat-inactivation process. The rate reaches a maximum in

100mM-phosphate and is unaffected by further additions of either phosphate or NaCl. To facilitate a comparison of the heat stabilities of the different genetic forms of the enzyme subsequent experiments were carried out in 100mM-phosphate buffer, pH 7.0, so that small differences in ionic composition between the various enzyme preparations were nullified. The results of such a comparison are shown in Fig. 2. In several experiments at temperatures between 50 and 65°C the type 2 enzyme consistently appeared more heat labile than the type 1 enzyme, with the type 2-1 enzyme preparations showing intermediate stability.

The rates of inactivation of types 1 and 2 enzyme preparations were measured at several temperatures. Arrhenius plots of the results (Fig. 3) gave the following values for the activation energy of the heat-inactivation process: 275.5 kJ/mol (65.9 kcal/mol) for the type 1 enzyme and 241.6 kJ/mol (57.8 kcal/mol) for the type 2 enzyme. These values support the earlier conclusion that the type 2 enzyme is less stable to heat than the type 1 enzyme.

The effect of electrolytes on the heat-inactivation process was investigated further. Rates of heat inactivation in the absence of electrolytes (i.e. in water) were measured at several temperatures on a purified sample of the type 1 enzyme that had been dialysed exhaustively against water. The Arrhenius plot of the results gave a value for the activation energy of the inactivation process of 430.5 kJ/mol (103 kcal/mol). This value is substantially higher than that obtained for the same enzyme in 100mM-phosphate buffer, pH 7.0, i.e. 275.4 kJ/mol (65.9 kcal/mol). Clearly the physical state of the enzyme, as reflected by its heat-stability properties, is greatly affected by changes in the ionic environment.

Discussion

Adenosine deaminase has been isolated from a variety of calf tissues, including serum (Cory *et al.*, 1967), spleen (Pfrogner, 1967a) and intestine (Brady & O'Connell, 1962). The enzyme has also been isolated from chicken duodenum (Hoagland & Fisher, 1967) and liver (Ma & Fisher, 1968). At the present time the metabolic role of the enzyme is still undefined, although several suggestions have been made: the enzyme may function as part of a pathway for purine salvage (Murray, 1971) or may possibly have a role in the control of blood flow by its action on adenosine, which is a vasodepressant (Conway & Cooke, 1939). The enzyme is frequently present in only small amounts (Phelan *et al.*, 1970) where it occurs in tissues, and for this reason it might be argued that the enzyme does not play a very significant role in metabolism. However, recent work with mice (Mustafa & Tewari, 1970) suggests that in some tissues a considerable part of the total activity of the enzyme is present in a latent form; previous estimates

of the amounts of this enzyme in various tissues may have to be revised.

Preparations of the enzyme from diverse sources have several properties in common but show marked differences in others. The physical properties show the greatest similarity in that most preparations of the enzyme have molecular weights in the region of 30000–50000, although forms with molecular weights of 100000 or greater have been reported (Ma & Fisher 1968; Akedo *et al.*, 1972). Irrespective of source the enzyme appears to be a fairly acidic protein with a pI value close to 5.0 (Murphy *et al.*, 1969; Pfrogner, 1967b). The human erythrocyte enzymes fit into this pattern, with molecular weights of 30000–50000 and pI values close to 5.0.

The enzyme prepared from a variety of sources appears to be relatively heat stable compared with other enzymes, and frequently temperatures greater than 60°C are required to appreciably inactivate the enzyme. There also appears to be a correlation between susceptibility to inactivation by heat and molecular size. Thus Ma & Fisher (1968) reported that the enzyme of molecular weight 100000 present in the tissues of several vertebrates that they studied was much more stable to heat than a form of the enzyme of molecular weight 30000 found in the same tissues. Similar results have been reported by Akedo *et al.* (1972) on the high- and low-molecular-weight forms of adenosine deaminase from human lung and stomach. The results reported here demonstrate the heat stability of the human erythrocyte enzymes. The further dramatic increase in stability in solutions of low ionic strength may be interpreted in terms of an association of enzyme molecules under these conditions to form a heat-stable polymeric species. Ma & Fisher (1968) obtained evidence for such a monomer-polymer relationship with the enzyme prepared from certain vertebrate tissues; in the presence of high concentrations of either $(\text{NH}_4)_2\text{SO}_4$ or sucrose the high-molecular-weight form of the enzyme dissociated into smaller species.

With regard to the catalytic properties of adenosine deaminase most preparations have pH optima close to 7.0 and K_m values for adenosine of 10–90 μM . Published values for the energy of activation for the enzymic hydrolysis of adenosine are in the range 16.7–37.6 kJ/mol (4–9 kcal/mol), except for the chicken duodenum (Hoagland & Fisher, 1967) and liver enzymes (Ma & Fisher, 1968), which give values close to 71 kJ/mol (17 kcal/mol). The pH optima of the erythrocyte enzymes is close to 7.0 and the values for K_m and activation energy for hydrolysis of adenosine also fall within the above range of values.

In spite of these similarities in basic catalytic properties there appear to be substantial differences between the active sites of enzymes from different sources as reflected by their differing responses to competitive inhibitors such as purine riboside and

inosine. Inosine, the product of the adenosine deaminase reaction, inhibits the enzyme from chicken duodenum (Hoagland & Fisher, 1967), from ox heart (Rockwell & Maguire, 1966) and from *Aspergillus oryzae* (Wolfenden, 1969). By contrast, the enzyme from calf intestine is not inhibited by inosine (Ronca & Zucchelli, 1968). Preliminary results on the kinetics of the human erythrocyte enzymes by using the integrated rate equation method of Foster & Niemann (1953) rule out the possibility of product inhibition. The erythrocyte enzymes further resemble the calf duodenum enzyme in that K_i values for inhibition by purine riboside are about $8\mu\text{M}$; almost identical values have been reported for the calf enzyme (Ronca & Zucchelli, 1968; Cory & Suhadolnik, 1965; Baer *et al.*, 1968). In marked contrast the enzyme from chicken duodenum gave a K_i value for this inhibitor approximately two orders of magnitude higher (Hoagland & Fisher, 1967).

In the present study on the properties of the different genetic forms of the human erythrocyte enzyme only one clear-cut difference between the variant forms was observed, namely a difference in heat stability. Similar studies on acid phosphatase from human erythrocytes, which exists in several inherited forms, have also failed to reveal clear-cut differences in properties between variants apart from differences in heat stability (Luffman & Harris, 1967; Scott, 1966; White & Butterworth, 1971). Even if the possibility of a correlation between the stability of the enzyme *in vitro* and *in vivo* is discounted the observed differences in heat stability presumably reflect differences in structure between the enzyme variants that may well be significant physiologically. Markert (1968) has argued that any difference in charge such as exists between the genetic variants of adenosine deaminase from human erythrocytes must result in differences in physiological functioning if only indirectly through secondary interactions with other cell constituents. Differences between the active sites of the variants may be extremely subtle and necessitate intensive studies with a wide range of alternative substrates and inhibitors of the enzyme.

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