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HUMAN ADENOSINE DEAMINASE

STOICHIOMETRY OF THE ADENOSINE DEAMINASE-BINDING PROTEIN COMPLEX

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Summary

In many human tissues adenosine deaminase exists as a large molecular weight complex (large form) composed of adenosine deaminase and an adenosine deaminase binding protein. The molar ratio of adenosine deaminase to binding protein in this large form complex appears to be 2 : 1, respectively, based on several observations. Scatchard-type analysis of the binding of ^{125}I-labeled adenosine deaminase to purified binding protein indicates that 2.15 mol of adenosine deaminase are bound to 1 mol of binding protein. Chemical cross-linking of ^{125}I-labeled adenosine deaminase-binding protein complex (large form) with glutaraldehyde produces 6 cross-linked species with molecular weights consistent with the proposed 2 to 1 stoichiometry. Sedimentation equilibrium analyses reveal a native molecular weight of 300 890 for the adenosine deaminase-binding protein complex (large form), 37 500 for small form adenosine deaminase, and 213 300 for the binding protein. A 2 : 1 molar ratio of adenosine deaminase and binding protein in the large form complex is most consistent with these molecular weight estimates.

Introduction

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) is widely distributed in human tissues and exists in multiple molecular and electrophoretic forms [1—9]. One form appears to be 'particulate' [5] while two soluble forms of adenosine deaminase have estimated molecular weights of 36 000—38 000 [3—5] (small form) and 222 000—298 000 [5,9] (large form) and are

interconvertible [5]. The small form adenosine deaminase has been purified to homogeneity from human erythrocytes and was shown (a) to be a single polypeptide with a molecular weight of 36 000—38 000, (b) to contain carbohydrate, and (c) to exhibit 3 distinct electrophoretic forms [3,4]. Physical and kinetic characteristics of the small form adenosine deaminase from several tissue sources appeared to be identical to the purified erythrocyte enzyme [3,5]. The small form of the enzyme can be converted to the large form of adenosine deaminase by incubation with tissue extract containing an excess of adenosine deaminase binding protein (formerly termed 'conversion factor' or complexing protein) [5,9,10,12]. The large form adenosine deaminase produced in this manner is physically and kinetically indistinguishable from the native form of adenosine deaminase normally present in that tissue extract [5,12]. Under appropriate conditions, this large form dissociates to the small form plus binding protein [5]. The adenosine deaminase binding protein purified from human kidney is a glycoprotein with a native molecular weight of 190 000—200 000 and a subunit molecular weight of 94 000 [9,12].

In this presentation, we analyze the stoichiometry of the adenosine deaminase-binding protein complex based on binding measurements, chemical cross-linking and sedimentation equilibrium analyses.

Experimental procedure

Materials

Bovine serum albumin, glutaraldehyde, hemoglobin, and 1,5-difluoro-2,4-dinitrobenzene were supplied by Sigma; 3—10% linear gradient rod polyacrylamide gels were obtained from Isolab Inc. Schwartz/Mann provided bromphenol blue while [8-^{14}C]adenosine (59 Ci/mol) was purchased from Amersham/Searle and Sephadex G-25 (medium) was obtained from Pharmacia. All other reagents used were of the highest purity commercially available.

Radioiodination of human erythrocyte adenosine deaminase

Human erythrocyte adenosine deaminase was purified to homogeneity by antibody affinity chromatography as previously described [3]. The highly purified enzyme yielded a single protein band by SDS polyacrylamide gel electrophoresis with a calculated molecular weight of 38 000 and a specific activity of 545 μmol · min^{-1} · mg^{-1}. Purified adenosine deaminase was radiolabeled with ^{125}I essentially as described by Hunter [13]. A mixture containing 10 μl of human erythrocyte adenosine deaminase (small form) (1 μg, 0.26 nmol), 1 μl Na^{125}I (1 mCi, 0.50 nmol), and 10 μl chloramine-T trihydrate (0.7 nmol) in 100 mM sodium phosphate pH 6.0 (buffer A) was shaken for 60 s at room temperature. The addition of 10 μl sodium metabisulfite (0.7 nmol) was used to stop the reaction. The ^{125}I-labeled reaction mixture was diluted with 40 μl of buffer A containing 2 mg/ml bovine serum albumin and 1 mM β-mercaptoethanol. The reaction mixture was then transferred quantitatively to a Sephadex G-25 (medium) column (0.7 × 28 cm). ^{125}I-labeled adenosine deaminase was eluted from the column with buffer A containing 0.5 mg/ml bovine serum albumin and 1 mM β-mercaptoethanol. Radioactivity appearing in the void volume was pooled and dialyzed 1 to 1000 v/v in buffer A containing 10%

glycerol. The ^{125}I-labeled adenosine deaminase was radioiodinated to an apparent specific radioactivity of 9.22 μCi/nmol. ^{125}I-labeled adenosine deaminase prepared in this manner remained stable for only 1.5 to 2 weeks at 4°C.

Radioiodination of human kidney adenosine deaminase binding protein

Human kidney adenosine deaminase binding protein was purified to homogeneity by adenosine deaminase affinity chromatography as previously described [12]. The purified protein revealed a single band by SDS polyacrylamide gel analysis with a calculated subunit molecular weight of 94 000. The protein was iodinated by the method above except the reaction buffer, column buffer, and dialysis buffer were all 100 mM sodium phosphate, pH 7.4. The ^{125}I-labeled binding protein was iodinated to an apparent specific radioactivity of 63.8 μCi/nmol. ^{125}I-labeled binding protein was stable for over 3 weeks at 4°C.

Polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was performed as previously described [3]. SDS-gel electrophoresis was performed using the buffer system of Laemmli [14]. Linear gradient (3—10%) cylindrical polyacrylamide gels (0.5—8.2 cm) (Isolabs Inc.) were pre-electrophoresed before use in 375 mM Tris-HCl, pH 8.9 containing 0.1% SDS. Gels were run until the bromphenol blue band just reached the bottom of the gel tube. In general gels were analyzed by slicing into 1.5 mm sections and measuring ^{125}I radioactivity.

Protein concentrations were determined by the method of Lowry et al. [15] using bovine serum albumin as standard.

Binding measurements

Reagents for the binding measurements were prepared as follows. Highly purified human erythrocyte adenosine deaminase (M_r = 38 000) was mixed with ^{125}I-labeled adenosine deaminase (small form) (2.02 · 10^{10} dpm/pmol) to yield a stock solution of ^{125}I-labeled adenosine deaminase (3.6 · 10^9 dpm/pmol, 0.454 μM) in 10 mM Tris-HCl, pH 7.4. Subsequent dilutions of this stock were made in the same buffer. Solutions of human adenosine deaminase binding protein (M_r = 213 000) were also prepared in 10 mM Tris-HCl, pH 7.4. To measure the extent of binding, 25 μl of adenosine deaminase binding protein (0.0514 μM or 0.1028 μM) was added to 10 μl of ^{125}I-labeled adenosine deaminase diluted serially from 0.454 μM to 0.056 μM. The reaction mixture was then incubated for 10 min at 37°C followed by the addition of 40 μl of a mixture containing 10% hemoglobin (20 mg/ml) and 10% bromphenol blue (3 mg/ml) in glycerol/water (1 : 1) to each reaction tube. The contents were blended in a Vortex mixer and quantitatively transferred to a 7.5% cylindrical polyacrylamide gel (0.7 × 7.5 cm) for electrophoresis at pH 8.9. The gel was electrophoresed and sliced at the visual hemoglobin band (R_F = 0.42). The gel section above the hemoglobin band (cathode end) containing exclusively ^{125}I-labeled adenosine deaminase-binding protein complex, termed large form adenosine deaminase, and the gel section below the hemoglobin band (anode end) containing exclusively unbound ^{125}I-labeled small form adenosine deaminase were individually analyzed for radioactivity in a Searle model 1190 gamma counter.

Cross-linking of ^{125}I-labeled adenosine deaminase-binding protein complex

Human adenosine deaminase was incubated with a stoichiometric quantity of ^{125}I-labeled binding protein to produce ^{125}I-labeled binding protein-adenosine deaminase complex. In addition, unlabeled binding protein was incubated with ^{125}I-labeled adenosine deaminase to produce a binding protein ^{125}I-labeled adenosine deaminase complex as described in the preceeding section. Cross-linking of the ^{125}I-labeled binding protein adenosine deaminase complex, binding protein-^{125}I-labeled adenosine deaminase complex, ^{125}I-labeled binding protein alone, and ^{125}I-labeled adenosine deaminase alone was performed at a final protein concentration of 0.1 mg/ml to favor intra over intermolecular cross-linking of subunits. All samples were dialyzed 1 to 1000 v/v for 24 h in the appropriate buffer and cross-linked with the following reagents.

Glutaraldehyde was used as a cross-linking reagent according to Kapoor and O'Brien [16] except the final concentration of glutaraldehyde used was 0.5% and the cross-linking buffer was 100 mM sodium phosphate, pH 6.8. Glutaraldehyde was incubated with each of the ^{125}I-labeled proteins individually for 1.5 and 24 h at room temperature. The reaction mixture was adjusted to a final concentration of 1% SDS and 5 mM β-mercaptoethanol and boiled for 5 min at 100°C. SDS-gel electrophoresis of the cross-linked samples was performed using 3—10% linear gradient cylindrical polyacrylamide gels and the buffer system of Laemmli [14]. Gels were sliced into 1.5 mm sections and analyzed for radioactivity as described above.

Cross-linking with dimethyl suberimidate was performed essentially by the method of Davies and Stark [17] while cross-linking with 1,5-difluoro-2,4-dinitrobenzene utilized the procedure of Baird and Hammes [18]. The reaction mixtures were prepared for SDS gel analysis and electrophoresed as described above.

Sedimentation equilibrium analysis

Sedimentation equilibrium analysis was performed with a Beckman AN-D rotor in a Beckman Model E analytical ultracentrifuge. All samples were dialyzed 1 to 1000 v/v for 24 h against 10 mM Tris-HCl, pH 7.4 containing 154 mM sodium chloride. Purified erythrocyte adenosine deaminase (0.525 mg/ml) was centrifuged at 34 000 rev./min against dialysate for 24 h at 20°C. Purified human kidney adenosine deaminase binding protein was centrifuged at protein concentrations of 0.63 and 0.32 mg/ml. Samples were centrifuged against dialysate at 13 000 rev./min for 24 h at 20°C. The adenosine deaminase-binding protein complex was prepared by incubating stoichiometric quantities of the two purified components together as described above. The sample (0.510 mg/ml) was centrifuged against dialysate at 10 000 rev./min for 24 h at 20°C. A partial specific volume of 0.725 gm/cc was assumed in the molecular weight calculations of binding protein and the adenosine deaminase-binding protein complex, while a calculated partial specific volume of 0.729 gm/cc was used in the molecular weight calculation of adenosine deaminase based on its amino acid composition [3].

Results

Binding measurements

Using in part, techniques previously developed for an adenosine deaminase binding protein assay [12], we have developed a quantitative assay to detect the binding of ^{125}I-labeled human adenosine deaminase to its binding protein. This binding reaction has been shown to be complete within 10 min at 37°C, to proceed over a broad pH range of 5 to 8 and to be unaffected by changes in ionic strength (0—200 mM KCl), azide (0.02%), thiols (5 mM), divalent metal ions (5 mM) and bovine serum albumin (1—25 mg/ml) [5]. After incubating ^{125}I-labeled adenosine deaminase and binding protein, the reaction mixture containing the ^{125}I-labeled adenosine deaminase-binding protein complex and free ^{125}I adenosine deaminase can be separated by electrophoresis on a 7.5% polyacrylamide gel, pH 8.9. As shown in Fig. 1, ^{125}I-labeled adenosine deaminase migrates with an R_F of 0.61 while the ^{125}I-labeled adenosine deaminase-binding protein complex migrates with an R_F of 0.14.

When a gel slice (R_F = 0.14, 1.5 mm) containing the ^{125}I-labeled adenosine deaminase-binding protein complex is layered on top of a new 7.5% polyacrylamide gel, electrophoresed as before, sliced, and analyzed for ^{125}I activity, greater than 97% of the total radioactivity applied is associated with the complexed form of adenosine deaminase. This high affinity suggests that the ^{125}I-labeled complex can be accurately quantified under the non-equilibrium conditions of polyacrylamide gel electrophoresis.

Binding of ^{125}I-labeled adenosine deaminase to binding protein is shown at two different binding protein concentrations (0.0514 μM and 0.102 μM) in
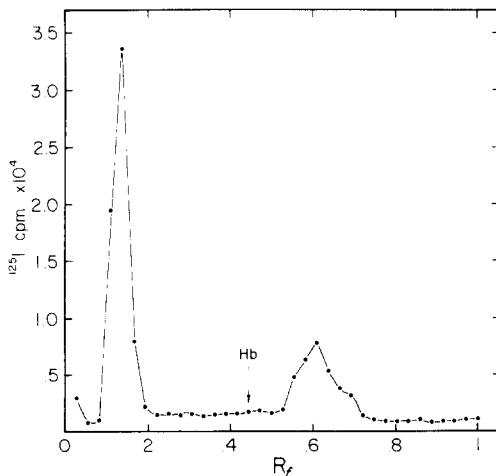

Fig. 1. Polyacrylamide gel electrophoresis of ^{125}I-labeled adenosine deaminase-binding protein complex (large form) and ^{125}I-labeled adenosine deaminase (small form). Purified adenosine deaminase binding protein was incubated with an excess of small form ^{125}I-labeled adenosine deaminase, and the reaction mixture was prepared and electrophoresed on a 7.5% polyacrylamide gel, pH 8.9 as described in Experimental procedure. The gel was sliced into 2 mm sections and analyzed for ^{125}I activity. The ^{125}I-labeled small form adenosine deaminase is represented by ^{125}I activity at R_f = 0.61 while ^{125}I-labeled large form adenosine deaminase migrates with an R_f = 0.14. Hemoglobin (Hb) was added as a visual marker (R_f = 0.42).
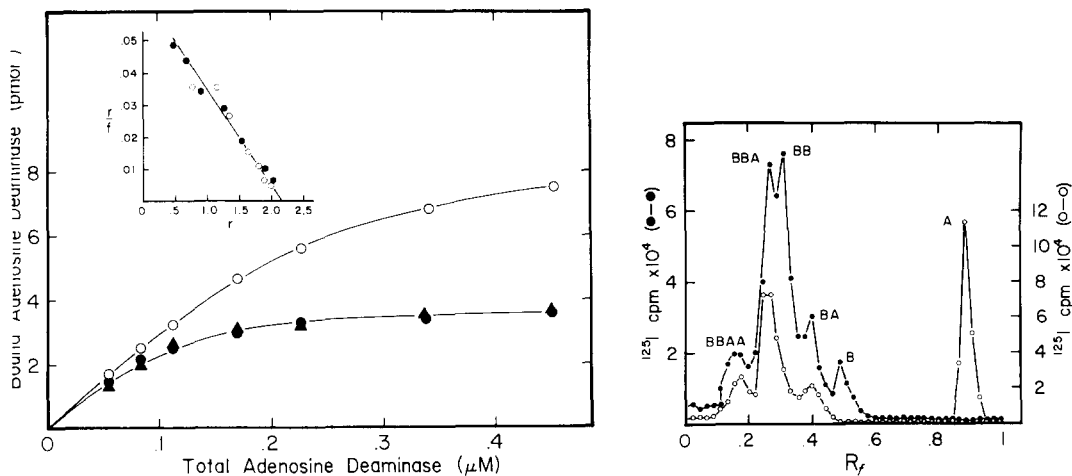
Fig. 2. Binding of human adenosine deaminase to binding protein. Highly purified adenosine deaminase binding protein (0.0514 μM, ●————●; 0.1028 μM, ————·) was added to ^{125}I-labeled adenosine deaminase (3.6 · 10[9] dpm/pmol) diluted serially from 0.454 μM to 0.056 μM as in Experimental procedure. ^{125}I-labeled adenosine deaminase (1.8 · 10[9] dpm/pmol), prepared by dilution with non-radioactive adenosine deaminase, was also incubated with 0.0514 μM of highly purified binding protein (▲————▲) in a similar manner. The ^{125}I-labeled adenosine deaminase-binding protein complex and ^{125}I-labeled adenosine deaminase were quantitated as described in Experimental procedure. The molecular weight of human erythrocyte adenosine deaminase (small form) and of human kidney binding protein used in these calculations was assumed to be 38 000 and 213 000, respectively, based on sedimentation equilibrium analyses data as shown in Results. The inset shows a Scatchard-type plot of these data in which r is the number of mol of small form adenosine deaminase bound per mole of binding protein and f is the concentration of free small form adenosine deaminase.

Fig. 3. SDS polyacrylamide gel profiles of the cross-linked ^{125}I-labeled adenosine deaminase-binding protein complex. Samples were cross-linked individually with glutaraldehyde for 1.5 h and electrophoresed on 3—10% linear gradient cylindrical SDS polyacrylamide gels as described in Experimental procedure. Gels were sliced into 1.5 mm sections and analyzed for ^{125}I radioactivity: cross-linked ^{125}I-labeled binding protein-adenosine deaminase complex (●————●) and cross-linked ^{125}I-labeled adenosine deaminase-binding protein complex (————·). The putative cross-linked species are indicated by the appropriate letter abbreviations.

Fig. 2. A Scatchard-type plot of these data (Fig. 2, inset) indicates that 2.15 mol of adenosine deaminase are bound to 1 mol of binding protein. When ^{125}I-labeled adenosine deaminase is diluted 100-fold with non-radioactive adenosine deaminase and reacted with binding protein (0.0514 μM) less than 3% of the radioactivity is associated with the adenosine deaminase-binding protein complex. In addition, when the specific radioactivity of ^{125}I-labeled adenosine deaminase is diluted 2-fold with non-radioactive adenosine deaminase and reacted with binding protein (0.0514 μM), the binding curve is not altered suggesting that radioactive and nonradioactive adenosine deaminase compete equally well for the same binding site on the binding protein (Fig. 2).

Cross-linking of the [125]*I-labeled adenosine deaminase-binding protein complex*

[125]I-labeled adenosine deaminase, ^{125}I-labeled binding protein, ^{125}I-labeled adenosine deaminase-binding protein complex and ^{125}I-labeled binding protein-adenosine deaminase complex were cross-linked individually with either glutaraldehyde, dimethyl suberimidate, or 1,5-difluoro-2,4-dinitrobenzene. [125]I-
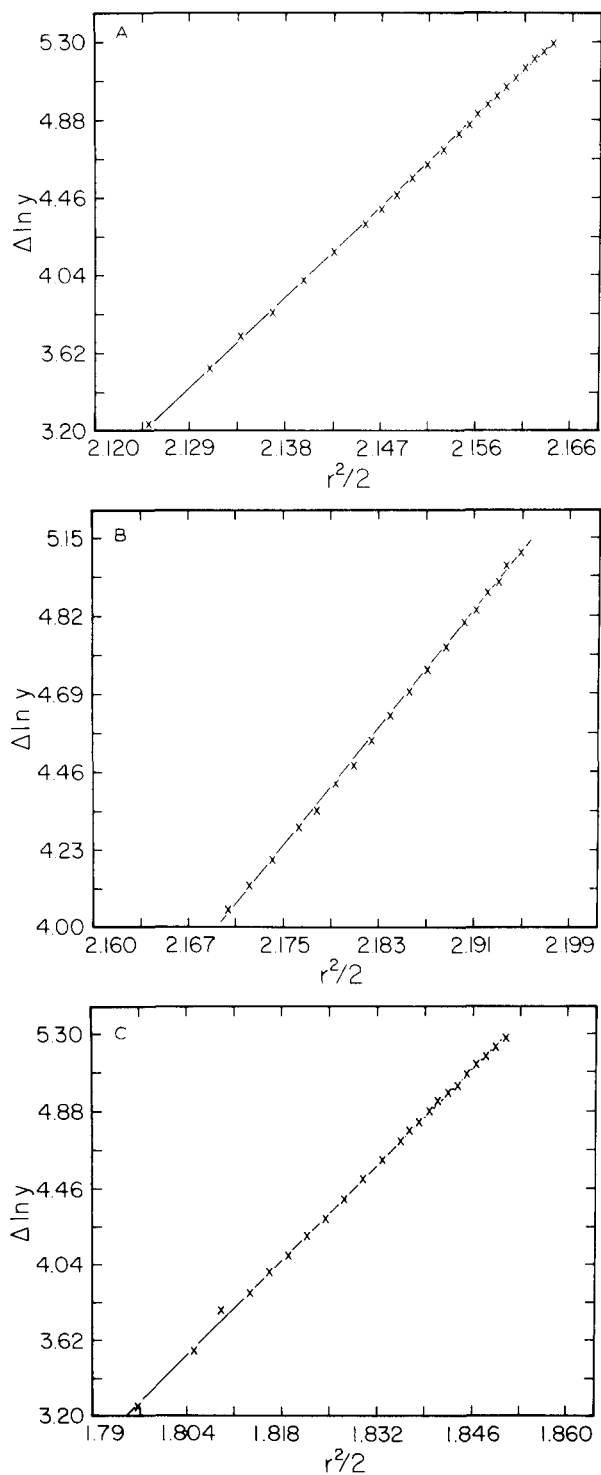
Fig. 4. Sedimentation equilibrium analysis. (a) Purified erythrocyte adenosine deaminase (0.52 mg/ml) was centrifuged at 34 000 rev./min against dialysate for 24 h at 20°C; (b) purified human kidney adenosine deaminase binding protein (0.63 mg/ml) was centrifuged against dialysate at 13 000 rev./min for 24 h at 20°C; (c) adenosine deaminase-binding protein complex (0.51 mg/ml) prepared 'in vitro' as described in Experimental procedure was centrifuged against dialysate at 10 000 rev./min for 24 h at 20°C.

labeled binding protein migrated with an R_F of 0.51 on a 3—10% linear SDS polyacrylamide gel and was radiochemically homogeneous. Cross-linking ^{125}I-labeled binding protein with glutaraldehyde resulted in two peaks, $R_F = 0.49$ and 0.33, corresponding to the cross-linked monomer (B) and dimer (BB), respectively. When ^{125}I-labeled binding protein-adenosine deaminase complex is cross-linked with glutaraldehyde, 5 radioactive peaks are consistently observed (Fig. 3, closed circles). Uncross-linked and glutaraldehyde cross-linked ^{125}I-labeled adenosine deaminase both resulted in only one peak of radioactivity at $R_F = 0.91$. When the ^{125}I-labeled adenosine deaminase-binding protein complex is cross-linked with glutaraldehyde, 4 radioactive peaks are observed (Fig. 3, open circles). Cross-linking for 1.5 or 24 h with glutaraldehyde led to no quantitative or qualitative change in the cross-linking patterns observed.

Cross-linking ^{125}I-labeled binding protein and ^{125}I-labeled binding protein-adenosine deaminase complex with dimethyl suberimidate resulted only in cross-linking between binding protein subunits. No other radioactive complexes were observed elsewhere on the SDS gel. Results of cross-linking ^{125}I-labeled adenosine deaminase and ^{125}I-labeled adenosine deaminase-binding protein complex with dimethyl suberimidate failed to produce any cross-linked radioactive species under the described reaction conditions. Likewise the reagent 1,5-difluoro-2,4-dinitrobenzene also failed to cross-link the adenosine deaminase-binding protein complex under the conditions described.

Sedimentation equilibrium analysis

Sedimentation equilibrium analysis was performed as described in Experimental procedure. As shown in Fig. 4a, b and c, all protein samples analyzed were apparently homogeneous as evidenced by a linear plot of $\ln \Delta y$ versus $r^2/2$. The following molecular weights were calculated from the slope of a linear plot of $\ln \Delta y$ versus $r^2/2$: human erythrocyte adenosine deaminase, $M_r = 37\,500$, human adenosine deaminase binding protein, $M_r = 213\,300$, and the adenosine deaminase-binding protein complex, $M_r = 300\,890$.

Discussion

Several observations confirm the specificity of ^{125}I-labeled adenosine deaminase binding. Firstly, ^{125}I-labeled adenosine deaminase, diluted 100-fold with non-radioactive adenosine deaminase in the binding reaction mixture, showed less than 3% incorporation of radioactivity into the adenosine deaminase-binding protein complex. Secondly, when the specific radioactivity of ^{125}I-labeled adenosine deaminase was diluted 2-fold with non-radioactive adenosine deaminase, no change in the extent of binding was observed suggesting that ^{125}I-labeled adenosine deaminase and non-radioactive adenosine deaminase compete for the same binding site with equal affinity. Although these binding measurements were not performed under equilibrium binding conditions the demonstrated stability of the isolated ^{125}I-labeled large form adenosine deaminase suggests that the complex can be accurately quantified under the conditions described. The binding reaction between purified human kidney adenosine deaminase binding protein and ^{125}I-labeled adenosine deaminase suggested that the large form complex was composed of 2.15 mol of adenosine deaminase and 1 mol of binding protein.

Another approach used to determine the stoichiometry of the large form adenosine deaminase complex was chemical cross-linking. The results of our cross-linking experiments clearly show that glutaraldehyde is a more effective cross-linker than either dimethyl suberimidate or 1,5-difluoro-2,4-dinitrobenzene. All of these cross-linkers tested react with free amino groups but differ in the distances over which they can bridge. 1,5-Difluoro-2,4-dinitrobenzene forms a maximum 3 Å bridge [18] while dimethyl suberimidate forms an 11 Å bridge between amino groups [17,19]. Glutaraldehyde, however, is not a distinct molecular species. In solution, glutaraldehyde has been reported to readily form homopolymers (α, β unsaturated aldehydes) of different lengths [19]. The use of this cross-linker then potentially allows more possible cross-links per molecule than the other reagents tested.

If large form adenosine deaminase were composed of 2 mol of adenosine deaminase (A,A) and 1 mol of binding protein (BB) then 8 possible cross-linked combinations would be theoretically possible: BBAA, BBA, BAA, BB, BA, B, AA and A. When the ^{125}I-labeled adenosine deaminase-binding protein complex and the ^{125}I-labeled binding protein-adenosine deaminase complex are cross-linked with glutaraldehyde, 6 different radioactive peaks are consistently observed (see Fig. 3). Radioactive peaks at R_F = 0.49 and 0.31 presumably corresponds to binding protein monomer (B) and dimer (BB), respectively, while the peak at R_F = 0.88 is most probably monomeric adenosine deaminase (A). The remaining radioactive peaks at R_F = 0.40, 0.27 and 0.18 appear to represent combinations of binding protein and adenosine deaminase subunits.

Since the mobility of each radioactive cross-linked species on SDS gels should roughly correlate to its molecular weight, this relationship can be used to limit the possible combinations of A and B subunits contained in the 3 coincident radioactive peaks present in Fig. 3. The radioactive peak at R_F = 0.40 which migrates between binding protein monomer (B, M_r = 106 000, R_F = 0.49) and dimer (BB, M_r = 213 000, R_F = 0.31) would presumably be a BA combination of expected molecular weight 144 000. The peak at R_F = 0.27 present just after the binding protein dimer peak (BB, M_r = 213 000, R_F = 0.31), could possibly be a BBA combination of expected molecular weight 251 000 and finally the peak at R_F = 0.18 could reflect the cross-linked native complex, BBAA of expected molecular weight 289 000. A plot of the expected molecular weight for the proposed cross-linked species versus the log of their R_F values was found to be linear. Noticeably absent in the radioactive gel profile are peaks corresponding to an AA species and a BAA species. This suggests that the distance between the two adenosine deaminase subunits in the native large form adenosine deaminase molecule is too great for successful cross-linking under these conditions. If the stoichiometry of large form adenosine deaminase were 1 mol of adenosine deaminase and 1 mole of binding protein then five cross-linked combinations of the subunits would theoretically be possible, two of which would contain A and B subunit combinations. Our data clearly shows more cross-linking than the 1 : 1 model would predict and thus strongly favors a minimum 2 : 1 stoichiometry model.

Sedimentation equilibrium analyses revealed native molecular weights of 37 500 for adenosine deaminase (small form), 213 300 for adenosine deaminase binding protein, and 300 890 for large form adenosine deaminase

(adenosine deaminase-binding protein complex). These molecular weight data would also be most consistent with the proposed stoichiometry of 2 mol of adenosine deaminase and 1 mol of binding protein for the large form complex.

The molecular weight of the adenosine deaminase-binding protein complex (large form) prepared 'in vitro' compares well with our previous estimated molecular weight of large form adenosine deaminase from several different tissue sources [5]. This suggests that the stoichiometry of the large form complex may be constant regardless of the tissue source of the enzyme.

Acknowledgements

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