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Studies on propylamine transfer activity in anti-AdoDATO antibodies

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Summary. Based on a structural similarity to the transition state of a propylamine transfer reaction involved in polyamine biosynthesis, S-adenosyl-(5′)-1,8-diamino-3-thiooctane (AdoDATO), the most potent inhibitor of spermidine synthases, was used as a hapten for mice immunization. From immunized mice sera, the IgG fractions were purified by means of affinity (protein A/G) chromatography. Sera and purified polyclonal antibodies from several mice were found to exert spermidine synthase-like activity. Moreover, by means of hybridoma technology, 19 anti-AdoDATO hybridoma clones have been screened for propylamine transfer activity and at least 6 were found to produce catalytic antibodies. These findings indicate the presence in the sera of active spermidine synthase-like catalytic antibodies. The reported results for the first time evidence the feasibility of preparation of N-alkylating antibodies, widening the biotechnological perspectives of antibodies as biocatalysts.

Keywords: Amino acids – Catalytic antibodies – Transition state analogs – Aminopropyl transferase – Polyamines

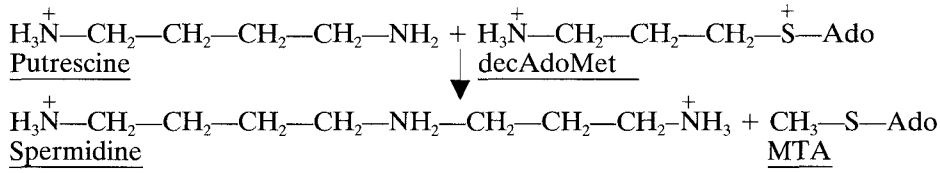
Abbreviations: AdoDATO: S-adenosyl-(5′)-1,8-diaminothiooctane; TSA: Transition state analog; decAdoMet: S-adenosyl-(5′)-3-methylthiopropylamine (decarboxylated adenosylmethionine); KLH: keyhole limpet hemocyanin; NMR: nuclear magnetic resonance; BSA: bovine serum albumine; WSC: 1-ethyl-3-(dimethylaminopropyl)carbodiimide (water-soluble carbodiimide); PBS: phosphate buffer saline; OPD: orthodiphenylenediamine; TCA: trichloroacetic acid; SDS-PAGE: sodium duodecylsulphate-polyacrylamide gel electrophoresis; SN2: bimolecular nucleophilic substitution; abzyme catalytic antibody; IgG: immunoglobulin G

Introduction

The obtainment of antibodies endowed with catalytic activities is now a well established and very promising achievement of biotechnology (Tramontano et al., 1986; Massey, 1987; Jacobs and Schultz, 1987). The main advantage of catalytic antibodies (abzymes) is that these proteins can be designed as catalysts on the basis of the mechanism known for the target reaction (Blackburn et al., 1989; Schultz et al., 1990). Many research groups are now trying to obtain "tailor-made" biocatalysts for many chemical reactions of biotechnological interest (Powell and Hansen, 1989).

The first step in abzyme preparation is the synthesis of a stable transition state analog (TS analog) involved in the reaction to be catalyzed; this analog is then used as a hapten for the preparation of potentially catalytic antibodies (Pollack et al., 1989). The design and synthesis of a suitable TS analog is often the most difficult task in the procedure. On the other hand, several TS analogs have already been synthesized in the past two decades in view of their potential activity as enzyme inhibitors (Wolfenden, 1987). In some instances these TS analogs have proved useful in the preparation of antibodies that exert catalytic activities similar to that of the target enzyme (Hilvert, 1988). The same strategy was used in the present paper.

One of the most studied TS analogs, the S-adenosyl-(5′)-1,8-diamino-3-thiooctane (AdoDATO, Fig. 1), shows indeed a very strong inhibitory activity towards the enzyme spermidine synthase, from eukaryotic as well as from prokaryotic sources (Pegg, 1982, Pontoni et al., 1984). Spermidine synthase catalyzes the transfer of an aminopropyl group from decarboxylated AdoMet (decAdoMet) to putrescine, yielding spermidine as a product (Scheme 1). Its



Scheme 1

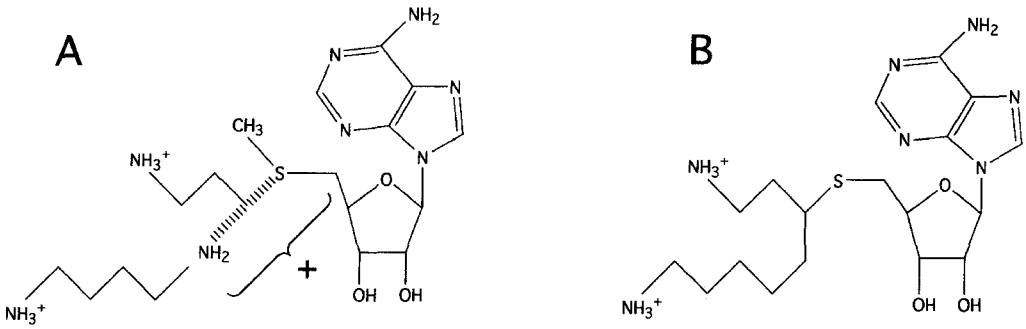


Fig. 1. A Structure of the hypothesized transition state of the propylamine transfer reaction catalyzed by spermidine synthase. The positive charge distribution over three atoms is illustrated. **B** Structure of S-adenosyl(5′)-1,8-diamino-3-thiooctane (AdoDATO), a stable analog of transition state A

kinetic mechanism has been deeply investigated by kinetic (Zappia et al., 1980) as well as by a stereochemical approach (Orr et al., 1988; Pontoni et al., 1983).

In this paper we describe the use of AdoDATO as a hapten to raise anti-AdoDATO polyclonal and monoclonal antibodies endowed with spermidine synthase activity. Spermidine synthase was envisioned as a good model system because its activity is not detectable in normal mouse serum; for this reason, the appearance of spermidine forming activity in sera by no means could be ascribed to any endogenous enzyme.

Materials and methods

Materials

AdoDATO was a kind gift of Prof. J. K. Coward of the Michigan State University at Ann Arbor, USA. [1,4^{14}C]putrescine and [1,4 ^{14}C]spermidine were purchase from Amersham, Bucks, U.K. All other chemicals were of the highest purity grade available from commercial sources.

Preparation of AdoDATO-protein conjugates

Conjugates of AdoDATO with keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were prepared by condensation of amide bonds between amino groups of AdoDATO and the carboxyl group of the two proteins by means of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, a water-soluble carbodiimide (WSC) as the condensing reagent. After 24 hours of incubation at room temperature under mild stirring conditions in glass vials, the separation of the conjugates from free AdoDATO was performed by means of Centricon 30 microconcentrators, cut-off 30,000. The presence of AdoDATO in the protein filtrate was monitored spectrophotometrically at λmax of AdoDATO, 259.4 nm. The final KLH-AdoDATO conjugate contained 21 nmol of analog per mg of protein; AdoDATO incorporation was 3.7%. The AdoDATO-BSA conjugate contained 30 nmol of analog per mg of protein; AdoDATO incorporation was 5%. The low conversions suggest that most of AdoDATO is bound only by a single amino group, as discussed below.

Polyclonal and monoclonal antibodies

Ten Balb/c mice, 6 to 8 weeks old, were immunized i.p. with either 50 μg (mice A, B, E, F, G, H, I, L) and 100 μg (mice C, D) of KLH-AdoDATO conjugate in complete Freund's adjuvant; the second dose, in incomplete Freund's adjuvant, was given on day 15. Each dose was given in PBS every 30 days, thereafter. High-titer anti-AdoDATO sera were obtained as judged by ELISA with BSA-AdoDATO conjugate. Blood samples were drawn after 4, 7 and 10 immunizations. These antisera were assayed as such for aminopropyltransferase activity and used for purification of IgG fraction as described below. The preparation of anti-AdoDATO IgG producing hybridomas was carried out according to Kohler and Milstein, and preliminarily screened by ELISA: 19 antibodies were found to bind AdoDATO.

ELISA assay

An enzyme immunoassay was developed to detect antibodies to AdoDATO. Briefly, polyvinyl plates (Nunc) were coated with 100 μl of BSA-AdoDATO conjugate (1 μg/ml) in 0.01 M carbonate-bicarbonate buffer, pH 9.7 and incubated 1.5 h at 37°C. A second

incubation was made with 300 μl of 5% BSA, 0.1% Tween 20 in PBS for 30′ at 37°C to block excess reactive sites. Into each well was then added a different serum dilution and incubation was performed for 1 h at 37°C. After 6 washes in PBS, peroxidase-conjugated rabbit anti-mouse (Dako) was added and incubated for 45′ at 37°C. The plates were washed 6 times in PBS, and OPD (ortho-diphenylen-diammine, Sorin) was added as substrate; 10 min later the reaction was stopped with H_2SO_4 1N. The plates were read at 492 nm with a Microplate Reader 3550 (BioRad).

Purification of IgG

IgG fractions were purified from mouse sera, hybridoma culture media and ascite fluids by means of protein A/G affinity chromatography kit (Pierce); the protein elution was monitored by means of absorbance at 280 nm. IgG solutions have been desalted on Presto Desalting columns (Pierce) and lyophylized, yielding 0.560 mg of purified IgG. SDS-PAG electrophoresis was run according to routine procedures, with 12.5% acrylamide in the running gel and 3% in the stacking gel, using Pharmacia low molecular weight standards, in a BioRad mini Protean electrophoresis apparatus. The coomassie-blue coloured gels showed only two electrophoretic bands at MW 50,000 and 25,000, assigned to heavy and light chain of immunoglobulins respectively.

Aminopropyltransferase assay

The standard incubation mixture contained: 3 nmol (732,000 dpm) [1,4^{14}C]putrescine or more, as specified; 56 nmol decAdoMet; 0.05 M TRIS/HCl buffer, pH 7.5; 40 μl of mouse serum (about 2.2 mg of total protein, ca. 0.19 mg of IgG), (as judged based on the amount of purified IgG obtained by affinity chromatography, taking conservatively into account a 70% yield), or 40 μl of 1.867 mg/ml purified IgG solution (total 0.0747 mg), or 40 μl of hybridoma culture medium (1.66 $\pm$ 0.40 mg of total proteins, ca. 100 μg IgG), or 40 μl of IgG purified from culture medium, as described below; and water up to 100 μl final volume (several controls were performed by adding non-immunized mouse sera, or antisera from mice immunized with non-AdoDATO bound KLH, instead of anti-AdoDATO antiserum). Incubation was carried out at 37°C for 2 hrs (1.5 hrs when initial velocity was strictly required); proteins were then precipitated by 25% TCA (10 μl) and centrifuged for 15 min. Finally, the supernatant was filtered off by means of Centricon 30 filters for 2 hrs, and 30 μl of filtrate were loaded onto a Varian LC-5000 HPLC equipped with an Ultrasil CX cation-exchange column, using 0.25 M phosphate buffer, pH 6.0 as the eluent, and a flow rate of 1 ml per min. The eluate was fraction-collected, added of 4 ml of Ready-Gel (Beckman) per fraction and the radioactivity was measured by means of a Beckman LS-7800 liquid scintillation counter. Under these conditions, the putrescine peak was centered at 7.5 min and the spermidine peak at 20 min. Alternatively, putrescine and spermidine were separated by TLC on silica-gel plates by means of BAWP 2:1:1:2 as the eluent. Under these conditions, Rf were 0.49 (putrescine) and 0.26 (spermidine). The relevant spots were evidenced by co-spotted carriers of cold polyamines, scraped, added of scintillation fluid and counted as described above. The dpm assigned to putrescine and spermidine chromatographic peaks allowed us to calculate the percent conversion of putrescine and hence to quantitate the amount of spermidine formed during the assay; as few as 5 pmol of spermidine formed could be detected unambiguously in all instances. Under the assay conditions, the estimated amount of endogenous putrescine in 40 μl of serum (<16 pmol) (Pegg, 1986) can be considered negligible. The possible formation of putrescine from spermidine via the polyamine oxydase pathway was also tested by adding labelled spermidine (219 pmol) instead of labelled putrescine, under the described assay conditions. Although the concentration of spermidine in this test was more than four times higher than in the assays, the amount of newly formed labelled putrescine was never detectable, hence its amount can be conservatively estimated as lower than 3 pmol per assay, a negligible

amount when compared to the 3nmol of labelled putrescine added in spermidine synthase assays, which by no means could affect the results.

Results

Sera from mice immunized with AdoDATO-KLH conjugate, as well as from untreated mice were tested for aminopropyltransferase activity (see Fig. 2). Controls included sera from the same mice under study before immunization and sera from mice immunized with different KLH-bound haptens. Sera from these control mice never presented any detectable spermidine formation under the assay conditions, thus ruling out the hypothesis of spermidine synthase contamination of positive serum samples. Blood samples were drawn after 4 and 7 immunizations: both withdrawals from mice B, E, F, G and L showed spermidine forming-acitivty (ranging from 1.943 to 21.59pmol of spermidine formed per mg of protein, when 30μM putrescine was used), sera from one withdrawal from mice D, H and I also showed detectable activity. In the remaining anti-AdoDATO antisera, no spermidine synthase-like catalysis was detected. In none of the assayed sera endogenous putrescine-forming activity from labelled spermidine was detectable under the assay conditions, hence the possibility of a significant interfering polyamine oxydase pathway was ruled out.

In order to directly relate the catalytic activity to the immunoglobulin fraction, the IgG fractions from sera from mice A, B, C and D, obtained after 10 immunizations, were purified by means of protein A/G affinity chromatography and tested by SDS-PAG electophoresis, which only showed bands of light and heavy immunoglobulin chains. IgG's from both catalytic sera B and

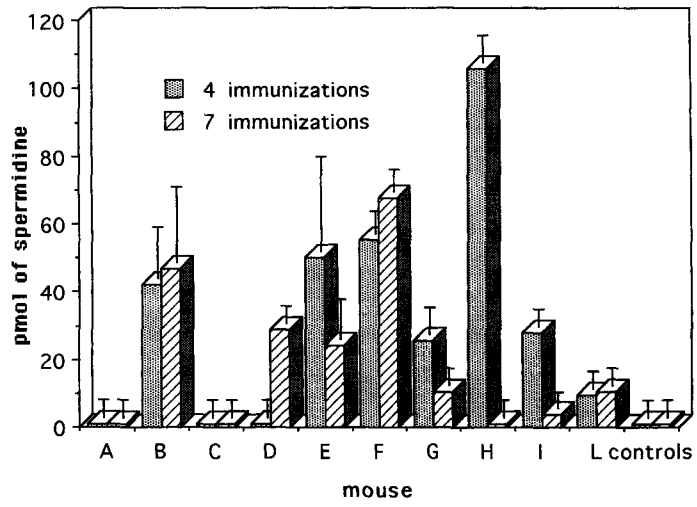


Fig. 2. Spermidine synthase-like activity directly detected in mouse sera after 4 and 7 immunizations with AdoDATO-KLH conjugate, as pmol of formed spermidine per assay as described under Materials and methods. Controls included several sera from mice immunized with non AdoDATO bound KLH; bound KLH

D were found to possess putrescine-dependent spermidine synthase activity (see Fig. 3), now with higher specific activity (68.27 and 185.94 pmol/mg).

The addition of 30 μM AdoDATO during the assays completely inhibited the catalytic activity, as expected because of the higher complementarity of abzyme(s) to the TS analog with respect to substrates.

By means of hybridoma technology, 19 anti-AdoDATO monoclonal antibodies producing clones have been prepared as judged by ELISA assays. The culture media of these clones have been screened using the propylamine

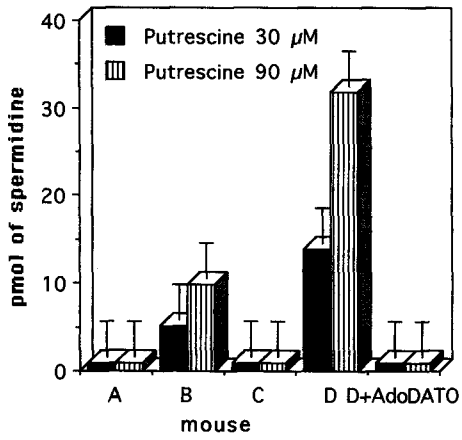


Fig. 3. Spermidine synthase-like activity detected in purified IgG's, from anti-AdoDATO mouse sera after 10 immunizations. In the last columns AdoDATO inhibition of abzyme activity is also illustrated

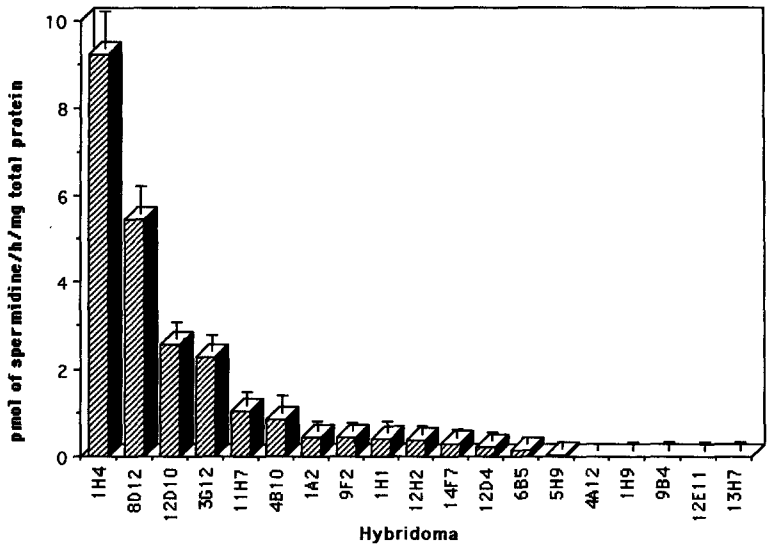


Fig. 4. Propylamine transferase-like activity in anti-AdoDATO antibody producing hybridoma culture media. The assays are as described under materials and methods, using 20 hours of incubation time to increase assay accuracy and number of turnovers. Whiskers indicate either standard deviation or sensitivity threshold, whichever is the lowest

transfer assay described under Materials and Methods. The results are illustrated in Fig. 4. At least 6 clones have been unambiguously identified as abzyme producing (1H4, 8D12, 12D10, 3G12, 11H7 and 4B10) and clones 1H4, 11H7, 1H1, 6B5 and 5H9 have been obtained as ascite fluids and the catalytic activity confirmed on IgG purified from ascite fluids from clones 1H4, 11H7 and 1H1 (data not shown). Using a conservative estimate of anti-AdoDATO antibodies in the media (5% of total IgG, Gallacher et al., 1992), in the total assay time can be measured, for example 324, 190, 86 and 75 turnovers for clones 1H4, 8D12, 12D10 and 3G12, respectively. These numbers clearly demonstrate that the antibodies can easily undergo many catalytic cycles and their behaviour is not of a stoichiometric type.

In order to characterize the kinetic behaviour of the abzyme catalyzed propylamine transfer reaction, always verified as Michaelian in all other reported abzymes, the activity of monoclonal antibody 1H4 has been studied as a function of putrescine concentration, when decAdoMet was kept constant (Fig. 5a), with a decrease of activity at high concentrations, that can be interpreted as putrescine excess inhibition in analogy with literature reports on other abzyme kinetics (Angeles et al., 1993). Conversely, the same inhibitory behaviour was not detected with respect to decAdoMet (Fig. 5b), at constant putrescine. It is interesting to note that propylamine transferring enzymes show the opposite substrate inhibition pattern, in that they are inhibited by high concentrations of decAdoMet and remain unaffected by high putrescine levels (Zappia et al., 1980). From the illustrated data, the enzymological constant of Table 1 can be calculated. The K_M values for putrescine are of the order of magnitude of that extrapolated for E. coli spermidine synthase. Based on the above mentioned estimate of anti-AdoDATO IgG concentration, the order of magnitude of k_{cat} can be calculated as 6.24×10^{-4} sec^{-1}. Consequently, an abzyme dependent reaction rate increase (as $k_{cat}/K_M/k_{uncat}$ ratio, Walsh, 1979) $\geq 8.7 \times 10^5$ can be estimated, demonstrating a very good efficiency of the antibody as a catalyst.

Discussion

AdoDATO has been synthesized in the laboratory of Prof. J. K. Coward as a TS analog, based on the catalytic mechanism of spermidine synthase as revealed by means of NMR as well as kinetic approaches (Orr et al., 1988; Pontoni et al., 1983; Zappia et al., 1980). To explain AdoDATO similarity to TS, it has been argued that the positive charge of TS, spread over three atoms in the TS (N-C-S), is more strictly mimicked by an uncharged structure (C-C-S) of AdoDATO rather than by cognate substructures bearing a localized charge as a sulfonium pole, that are in this respect more similar to the decAdoMet substrate (Zappia et al., 1980). Moreover, AdoDATO can also be simply envisioned as a bisubstrate analog, and hence its complementary antibody, by recognizing sites of both substrates matched by the analog, might function as an entropy trapy (Blackburn et al., 1989, 1991; Wolfenden, 1987). For all these reasons, it was reasonable to envision AdoDATO as a suitable

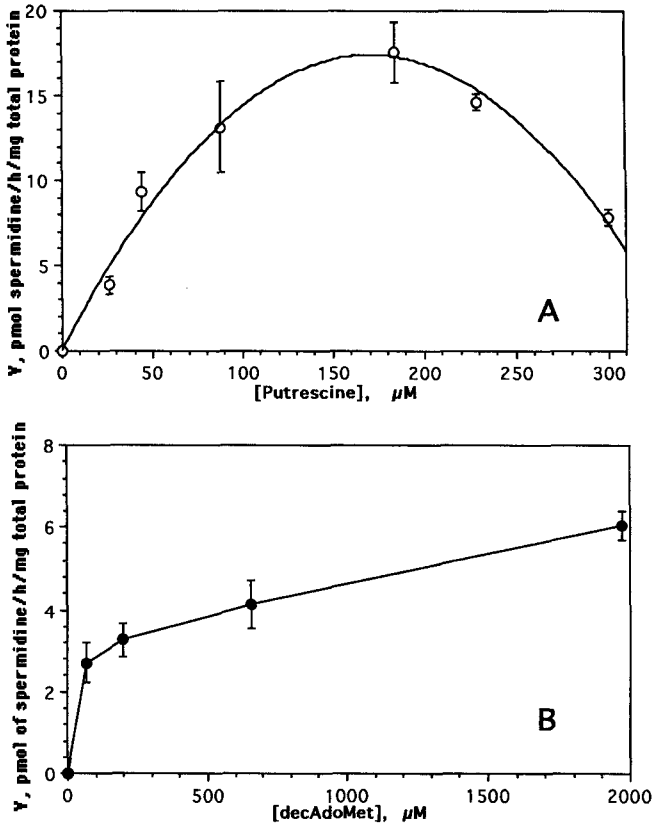


Fig. 5. A Putrescine dependent propylamine transferase activity in monoclonal antibody 1H4. Assays are performed as described under Material and methods and decAdoMet is kept constant at 560μM. Whiskers indicate the standard deviation or the sensitivity threshold, whichever is the lowest. **B** decAdoMet dependent propylamine transferase activity in monoclonal antibody 1H4. Assays are performed as in A, with 30μM constant putrescine. Whiskers indicate the standard deviation or the sensitivity threshold, whichever is the lowest

Table 1. Enzymological constants calculated for mAb 1H4

K'_M (putrescine)	83 ± 40μM
V'_{MAX}	16 ± 7μM
k'_{cat}	$\approx 6.24 \times 10^{-4}$ sec^{-1}
k_{uncat}	$\leq 2.6 \times 10^{-11}$
$k'_{cat}/K_M/k_{uncat}$	$\geq 2.8 \times 10^5$

These enzymological constants have been evaluated at decAdoMet 560μM and under the conditions specified in Fig. 5. k_{uncat} was estimated at 56μM decAdoMet and 80μM putrescine. See also the text under "Results" for further explanations.

hapten for stimulating catalytic antibodies. It is worth noting that this is the first description of antibodies catalyzing alkyl transfers onto a nitrogen atom, a SN2 reaction. Based on the very good catalytic efficiency of antibody 1H4 ($\geq 10^5$ rate increase), the possibility of preparing antibodies catalyzing other

N-alkylation reactions by means of the same kind of TS structural mimicking is currently under study.

The perspective of eliciting active abzymes in the blood is very promising, in that it might be exploited in stimulating the *in vivo* appearance of catalytic activities of metabolic and/or therapeutic relevance. The results here reported are consistent with the hypothesis that antibodies endowed with aminopropyltransferase activity are indeed present in some of the anti-AdoDATO antisera. No spermidine formation was detected in assays performed with control mouse sera, or with hybridoma culture media, thus indicating that enzyme-like activity should be ascribed to the immunoglobulin fraction. Moreover, our antibodies maintained their activity after purification from mouse sera or culture media, thus directly relating the spermidine forming activity to IgG fraction. The hypothesis of contamination of positive samples with spermidine synthase traces is not consistent i) with the absolute lack of detectable enzyme activity in control normal mouse sera, which is also supported by literature reports (Cacciapuoti et al., 1991), ii) with the lack of activity in control sera from mice immunized with other KLH-bound haptens, and iii) with the different inhibition pattern evidenced by monoclonals with respect to the propylamine transferring enzymes.

Owing to the lack of enzyme activity in mouse serum, our model system can easily be exploited for studies on the variability of catalytic behaviour of abzymes with the time during the development of the immune response. The results shown in Fig. 2 illustrate in some cases a wide fickleness of the catalytic activity at the two different times. No studies are available in literature on the subject, although this information would be very helpful in choosing the best time to kill the immunized mice. More accurate studies are necessary in this respect, though, because the catalytic efficiency of antibodies is not necessarily enhanced as a consequence of the improvement of antibody to antigen binding carried out during the immune response: over-refinements of TS analog complementarity may also theorethically result in a lesser strict complementarity to the true TS (which anyhow differs from its analog) that remains the ultimate target of complementarity of a catalytic protein.

In order of verify the interesting perspective of a possible use of endogenously triggered catalytic antibodies in metabolic engineering, the possible *in vivo* effects on the polyamine pool will be studied. In this respect it is worth emphasizing the versatility of the TS analog, AdoDATO, already proven to inhibit spermidine synthase in vivo when administered as such, and now found able of inducing an exactly opposite effect such as the triggering of antibody dependent spermidine synthase-like activity, when used as a hapten.

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