DETERMINATION OF THE RATES OF SYNTHESIS AND DEGRADATION OF ENZYMES BY COMPLEX FORMATION WITH RADIOACTIVE LIGANDS

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1. Introduction

An alternative approach to the immunoprecipitation procedure for the determination of the rates of synthesis and degradation of enzymes [1] is presented. The method is based on the determination of the molar specific radioactivity of the purified ^{14}C-labeled enzyme. This is done by saturating the ^{14}C-labeled enzyme with a stoichiometrically binding ^{3}H-labeled ligand of known specific radioactivity ('radioaffinity labeling'). The amount of bound ^{3}H-label thus indicates the molar amount of isolated enzyme and the ratio of the ^{14}C/^{3}H-radioactivity indicates the extend of labeling per mole of enzyme (specific radioactivity). The method is limited to enzymes for which stoichiometrically binding ligands are available. Dihydrofolate reductase was used to illustrate the method.

2. Materials and methods

The derivation and propagation procedures for the methotrexate-resistant BHK subline A5 have been described [2,3]. The cells were grown in methotrexate-free medium for at least 10 generations prior to the labeling experiments. Pulse-labeling was performed by supplementing the medium with a uniformly labeled ^{14}C-amino acid mixture (New England Nuclear, Boston Mass.).

Pulse-labeled dihydrofolate reductase (EC 1.5.1.3) was isolated as previously [3] except that 5×10^6 cells were lysed in 0.1 ml of hypotonic buffer and 0.1 ml of lysis buffer. The high speed supernatant of the lysate was loaded directly onto a 3 × 30 mm affinity column containing 50 μl of packed methotrexate-Sepharose. The column was washed sequentially 5 times with 0.2 ml of 50 mM phosphate, pH 5.8, then 5 times with 0.2 ml of 5 mM ammonium carbonate, and finally 4 times with 50 μl of 1 mM folate in 5 mM ammonium carbonate. The enzyme was eluted in the last four fractions.

For complex formation the enzyme fractions were pooled, mixed with 5 μl of [^{3}H]methotrexate, 10 μl of 10 mM NADPH, and 20 μl of glycerol and applied to the top of a soluble 7% acrylamide gel containing 0.7% of *N,N'*-diallyltartardiamide in 100 mM imidazole–HCl, pH 6.8. Electrophoresis was carried out as described [3]. Following electrophoresis, the gel was cut into 1 mm pieces and each piece digested with 100 μl of 0.1 N periodic acid. They were then neutralized with 100 μl of 0.15 N Tris and counted in 10 ml of scintillation fluid containing 25% of Triton X-100 and 5% of water. [^{3}H]methotrexate (Amersham/Searle, Chicago Ill.) was diluted with nonlabeled methotrexate (gift of Lederle, Pearl River, N.Y.) and purified on DEAE-cellulose [2].

3. Results

3.1. *Binding of [^{3}H]methotrexate to dihydrofolate reductase*

Dihydrofolate reductase (EC 1.5.1.3) from a variety of sources has high affinity for the inhibitor methotrexate (K_{diss} 10^{-8} to 10^{-11} M) [4]. The binding of the inhibitor has been exploited in numerous studies to purify the enzyme by affinity chromato-

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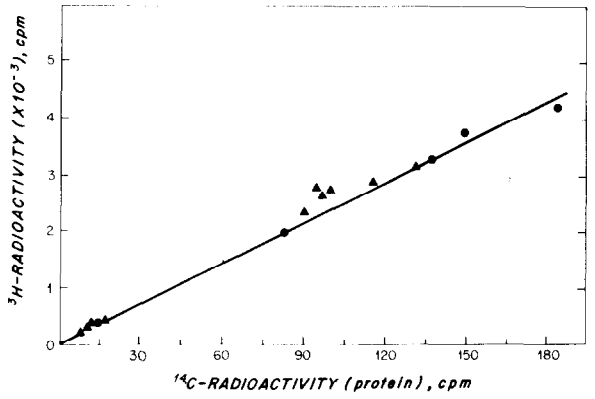

Fig.1. Linearity of [^{3}H]methotrexate binding to dihydrofolate reductase. The enzyme was isolated as described in Methods from hamster cells which had been grown for 3 days in medium containing 0.1 μCi/ml of [^{14}C]leucine (0.25 mCi/mmol). Various amounts of labeled enzyme (0.2 to 4.3 mg corresponding to 9 to 190 pmoles, ref. [2]) were mixed with 1.5 nmol of [^{3}H]methotrexate (67 900 dpm/nmol) and applied to gels for electrophoresis. The region of the gels containing the complexes were cut into pieces and counted for the ^{3}H- and ^{14}C-radioactivity. ▲, Form I; ●, Form II of hamster cell dihydrofolate reductase.

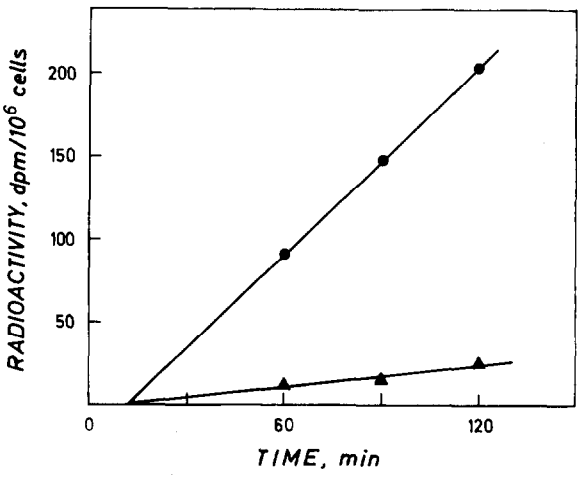

Fig.2. Rates of synthesis of dihydrofolate reductase. Cells were grown for the periods indicated in normal medium supplemented with 2.5 μCi/ml of ^{14}C-labeled amino acids. Pulse-labeled dihydrofolate reductase was isolated by affinity chromatography, mixed with [^{3}H]methotrexate (62 000 dpm/nmol) and processed as described in Methods. ▲, rate of synthesis of Form I; ●, rate of synthesis of Form II of hamster cell dihydrofolate reductase (dpm/h/10^6 cells).

graphy or to titrate its activity. To examine the extent and linearity of the binding, known amounts of ^{14}C-labeled enzyme were mixed with [^{3}H]methotrexate and applied to acrylamide gels. The two forms of hamster cell dihydrofolate reductase [3] and the excess inhibitor were separated by electrophoresis. The amounts of [^{3}H]methotrexate bound to both forms of the enzyme were directly proportional to the amounts of the two forms (fig.1). From the specific radioactivities it was evident that a stoichiometric (1:1) complex was formed between enzyme and ligand. In 19 assays up to 550 pmol of enzyme were tested. The standard deviation from the expected 1:1 relationship was 10%. Due to increased counting errors the deviation increased to 15% at lower enzyme levels (below 20 pmol).

3.2. *Determination of the rate of synthesis*

[^{3}H]Methotrexate binding has been used for the determination of the rate of synthesis of dihydrofolate reductase (fig.2). ^{14}C-pulse-labeled enzyme was isolated by affinity chromatography. The two forms were separated and quantitated by [^{3}H]methotrexate binding as before. Because of the two different isotopes used, the specific radioactivities (measured as ^{14}C-dpm/mol of bound [^{3}H]methotrexate = dpm/mol of enzyme) could be determined in a single experiment. The values obtained for the different labeling periods were multiplied by the steady state concentrations of the two enzyme forms in 10^6 cells (33 pmol of Form II and 3.3 pmol of Form I, ref. [5]) to obtain the rates of synthesis per 10^6 cells (fig.2). In this particular experiment (2.5 μCi/ml of ^{14}C-labeled amino acids) the rates were 13 dpm/h/10^6 cells for Form I and 114 dpm/h/10^6 cells for Form II. From other experiments in which the specific radioactivity of newly synthesized cytoplasmatic proteins was also measured (dpm/mg), it was calculated that these rates of synthesis corresponded to the synthesis of 6 ng of Form I and 63 ng of Form II of the reductase per hour per 10^6 cells [5].

The lag in enzyme synthesis between 0 and 15 min which is often observed can be explained by the time needed for the cells and the culture medium to equilibrate with the CO_2 in the incubator and to reach the incubation temperature of 37°C.

4. Discussion

A major advantage of the radioaffinity labeling approach for the determination of the rates of synthesis or degradation of enzymes is that monospecific antibodies are not required. Furthermore there exist already a large number of high affinity ligands which are used in affinity chromatography [6]. Examples are biotin [7], FdUrd [8,9], nicotinamide-5-bromoacetyl-4-methyl-imidazole dinucleotide [10], or estradiol [11]. Probably these ligands could be used to determine the turnover rates of avidine, thymidilate synthetase, dehydrogenases, and even estrogen receptors.

Affinity chromatography has been used previously for the determination of turnover rates of enzymes [12], but poor reproducibility of enzyme yields has limited use of the method. Combination with the double isotope technique of Arias circumvents some of the difficulties [13], but the method is still generally limited to the determination of *relative* rates, i.e., rates before and after enzyme induction. This limitation does not apply to the radioaffinity labeling technique, because with this method specific radioactivities and absolute values are obtained. Also this method is independent of enzyme yields or purification artefacts; and since it is simple and fast, multiple time points can be obtained easily.

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