DETERMINATION OF REACTIVE GROUPS OF POLYMER CARRIERS WITH AMINO ACID 4-NITROANILIDES*

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Received 18 July 1972

1. Introduction

Carrier bound biologically active substances (e.g. enzymes, enzyme effectors) have attracted great interest for preparative and analytical application in biochemistry [1, 2]. They are prepared by using insoluble hydrophilic natural or synthetic polymers with free reactive groups, e.g. anhydride-, azide-, cyanate-, diazonium-, epoxide-, vinyl-groups.

The choice of suitable reactive groups and of optimum conditions for the binding reaction is difficult since methods for the quantitative determination of bound enzymes or effectors e.g. measurement of activity or affinity, titration of reactive groups, elementary analysis are time consuming and insufficient. In this respect we found that the use of amino acid 4-nitroanilides is very favourable. It is possible to bind the amino acid 4-nitroanilides like proteins or peptides to the active groups of the polymers, e.g. by means of the free amino group. Amino acid 4-nitroanilides, therefore like amino acids or dipeptides [3] are useful model substances for proteins provided that factors such as steric hindrance influence of lipophilic or hydrophilic sites are not to be measured. The particular advantage in using amino acid 4-nitroanilides is the easy quantification: after alkaline hydrolysis 4-nitroaniline can be directly determined photometrically [4, 6].

* The results have partly been presented in a lecture presented to GDCh, Ortsverband Darmstadt, January 14, 1972.

Abbreviations:

Ala-PA = L-alanine 4-nitroanilide;
Ac-Tyr-PA = *N*-acetyl-L-tyrosine 4-nitroanilide;
B-A-PA = N^{α}-benzoyl-D,L-arginine 4-nitroanilide;
B-Lys-PA = N^{α}-benzoyl-L-lysine 4-nitroanilide;
Glu-Phe-PA = *N*-glutaryl-L-phenylalanine 4-nitroanilide.

2. Materials and methods

The reactive carriers applied have been prepared by the chemical department of E. Merck, Darmstadt. The amino acid 4-nitroanilides are commercial products of E. Merck, Darmstadt.

2.1. *Assay of binding carrier groups*

The amino acid 4-nitroanilide, dissolved in 0.1 M phosphate buffer of appropriate pH, is incubated with 10—100 mg active carrier for at least 1 hr. The conjugate is separated and washed by suction, until no amino acid 4-nitroanilide can be detected in the final washings by means of hydrolysis with 1 N NaOH. The conjugate suspended in 10 ml water is hydrolyzed for 30 min at 60° after addition of 6 ml 1 N NaOH. After neutralization the volume is brought to 100 ml with 0.1 phosphate buffer, pH 7. The insoluble carrier is separated and the concentration of free 4-nitroaniline is measured photometrically at 405 nm (ϵ_{405} 1 cm = 9620 cm^2 $mmole^{-1}$ [4]).

3. Results

3.1. *Applications of the method*

3.1.1. Determination of the binding capacity of carriers

The capacity of cellulose derivative with active aldehyde groups is determined by incubation of the carrier with increasing concentrations of Ala-PA (table 1).

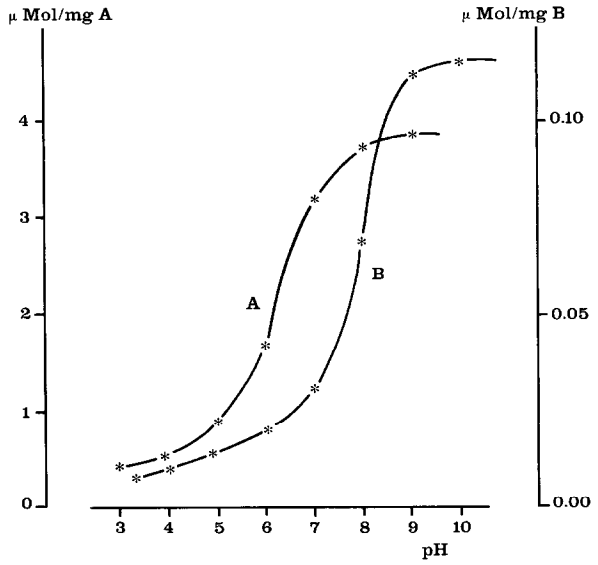

Fig. 1. pH-dependence of the binding reaction. Cross-linked copolymer of maleic acid B-Lys-PA, 5 mM; 25°; incubation time 1 hr. Copolymer with epoxide groups (B); Ala-PA, 5 mM; incubation time 3 hr.

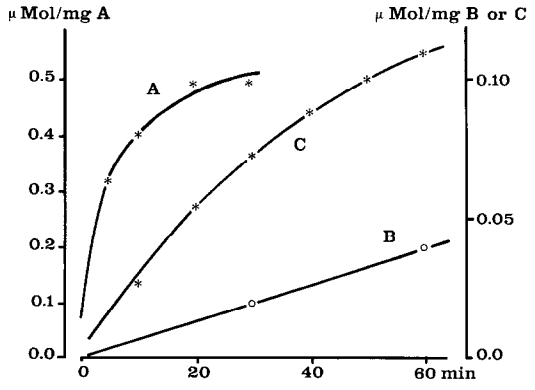

Fig. 2. Time course of binding reaction in 0.1 M phosphate buffer at 25°. A) Cross-linked copolymer of maleic acid anhydride; Ala-PA, 1 mM; pH 8.0. B) Copolymer with epoxide groups; Ala-PA, 5 mM; pH 9.0. C) CM-cellulose acid azide; Ala-PA, 0.5 mM; pH 9.0.

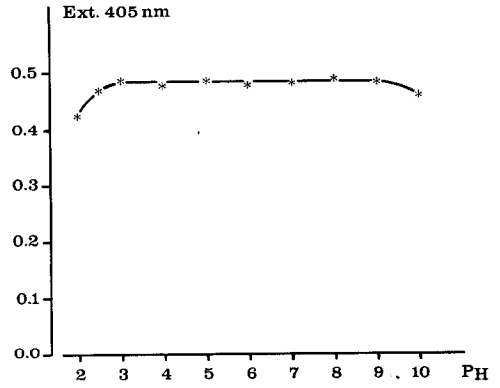

Fig. 3. pH-dependence of the extinction coefficient of 4-nitroaniline. 4-Nitroaniline is dissolved 50 μM in 0.1 M buffer containing: citrate, pH 2.0–4.0; phosphate, pH 5.0–8.0; borate, pH 9.0–10.0.

Table 1
Dependence of binding reaction on concentration.

Concentration (mM Ala-PA)	μmole Ala-PA bound per g carrier
1	75
2	110
5	130
10	150
20	160
40	155

50 mg cellulose derivative with reactive aldehyde groups were incubated for 70 min with Ala-PA in 40 ml 0.1 M phosphate buffer pH 9.0 at 25°.

3.1.2. pH-dependence of the binding reaction

In general, the pH determines the reaction rate and the amount of substance bound as well as the extent of interfering side reactions. Moreover, enzymes are stable only within a limited pH range. Preliminary investigations with amino acid 4-nitroanilides enable the determination of pH dependence of the binding reaction. The amounts of B-Lys-PA and Ala-PA fixed at different pH values to cross-linked maleic acid anhydride and a copolymer with reactive epoxide-groups respectively, are shown in fig. 1.

Side reactions may be measured by incubating the reactive carrier in buffer and adding the amino acid 4-nitroanilide at various times.

3.1.3. Determination of time dependence

Fig. 2 shows the time course of the coupling of Ala-PA to three insoluble carriers with different reactive groups. The reactivity of the epoxide groups (fig. 2, curve B) is low, so that only substances incubated at high molar concentration, are bound to a sufficient degree. Polymers with anhydride or azide groups couple Ala-PA rapidly. However, as in the case of cyanate groups [3], side reactions, such as hydrolysis, result in the binding of smaller amounts of Ala-PA than theoretically anticipated (7 and 3.2 μmole/mg, respectively [5]).

4. Discussion

At present 4-nitroanilides of various amino acids are mainly used as substrates for proteinases [4–7]. In comparison to other amino acid derivatives they are also favourably utilized in the method of analysis described above. Particularly the sufficient stability in basic or acidic solutions, and the high molecular extinction of 4-nitroaniline, which is constant within a wide range of pH values (fig. 3) are of great value in this respect.

Considering the above mentioned properties the amino acid 4-nitroanilides are very useful reagents for the determination of:

1. the coupling capacity of reactive carriers
2. the reactivity of binding groups and
3. the influence of various conditions on the reaction.

Using 4-nitroanilides of different amino acids the applicability of the described method is favourably enhanced. Different reactive groups of carriers can be examined on their binding properties: e.g. diazonium groups with Ac-Tyr-PA, reactive groups for guanidyl residues with B-A-PA, amino groups (of carrier) with Glu-Phe-PA.

References

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