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Colorimetric Determination of Reactive Amino Groups of a Solid Support Using Traut's and Ellman's Reagents

T. T. NGO

BioProbe International Inc., 2842 C Walnut Avenue, Tustin, CA 92680 USA

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ABSTRACT

A simple colorimetric method has been developed for the quantitative determination of reactive amino groups of a solid support. The method calls for the use of a reagent that reacts specifically and facilely with amino groups and concurrently introduces free sulfhydryl groups. The latter can then be titrated colorimetrically by using the well-known Ellman's reagent. Thus, an excess of 2-iminothiolane (Traut's reagent) was reacted with amine-carrying solid supports. After removing unreacted 2-iminothiolane and keeping the resultant solid-phase sulfhydryl groups in a reduced state by using dithiothreitol, the solid supports, after being thoroughly washed, were then reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) to quantify the sulfhydryl groups that were generated from reacting solid-phase amino groups with Traut's reagents.

Index Entries: Colorimetric determination, of reactive amino groups; reactive amino groups, colorimetric determination of; Traut's reagent; Ellman's reagent; solid support, reactive amino groups of; amine-carrying solid supports.

INTRODUCTION

Optimizing the conditions for coupling ligands to an amine-carrying solid support will be significantly simplified if the number of reactive amino groups of the solid support is known in advance. The number of

*Author to whom all correspondence and reprints requests should be addressed.

reactive amino groups of a solid support depends on their total number, accessibility, and microenvironments.

At least three methods have been developed for determining the reactive amino groups of a solid support. In one method the amine-carrying support is treated with a solution of picric acid to form a salt with the solid-supported amino groups (*1,2*). After washing away the excess picric acid, the support is treated with a strong base to release the picrate from the support into solution and its concentration is then quantified spectrophotometrically. The second method is based on the condensation of 2-hydroxy-1-naphthaldehyde with the solid-supported amino groups (*3,4*). After the formation of aldimines, the condensation products, and washing off the excess reagents, the chromophores are displaced from the support by reacting the solid support with amines, and the concentration of the displaced aldimine in the soluble phase is subsequently measured spectrophotometrically. The third method used an acylating reagent, such as *N*-succinimidyl-3-(2-pyridyldithio) propionate to react with solid-supported amino groups (*5*). After removing the excess acylating reagents, the support was thoroughly washed and treated with a reducing agent, such as dithiothreitol (DTT), to quantitatively release pyridine-2-thione from the solid support to the solution, and the concentration of the displaced pyridine-2-thione in the solution phase was measured in a spectrophotometer.

The above cited methods for measuring reactive amino groups of a solid support are not entirely satisfactory because the reagents used in these methods react not only with amino groups, but also with other functional groups. It is therefore desirable to have a method that uses reagents that react specifically with amino groups.

In this communication I introduce a new, convenient, and specific colorimetric method for measuring the reactive amino groups of a solid support. This method uses two readily available reagents: (1) Traut's reagent, 2-iminothiolane (ITL), which reacts specifically with amino groups (*6–8*) and (2) a thiol specific reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which is known as Ellman's reagent (*9*).

PRINCIPLE

Chemical reactions used in the development of a specific, colorimetric method for the determination of the reactive amino groups of a solid support are presented in Fig. 1. Amine-carrying solid supports were suspended either in dry ethanol or in a bicarbonate buffer, pH 8.5, and were reacted with excess ITL. Under slightly alkaline conditions, ITL reacts smoothly, rapidly, and specifically with amino groups (Reaction 1, Fig. 1). The consequence of this reaction is the generation of, on the solid support, one sulfhydryl group per one amino group reacted with the rea-

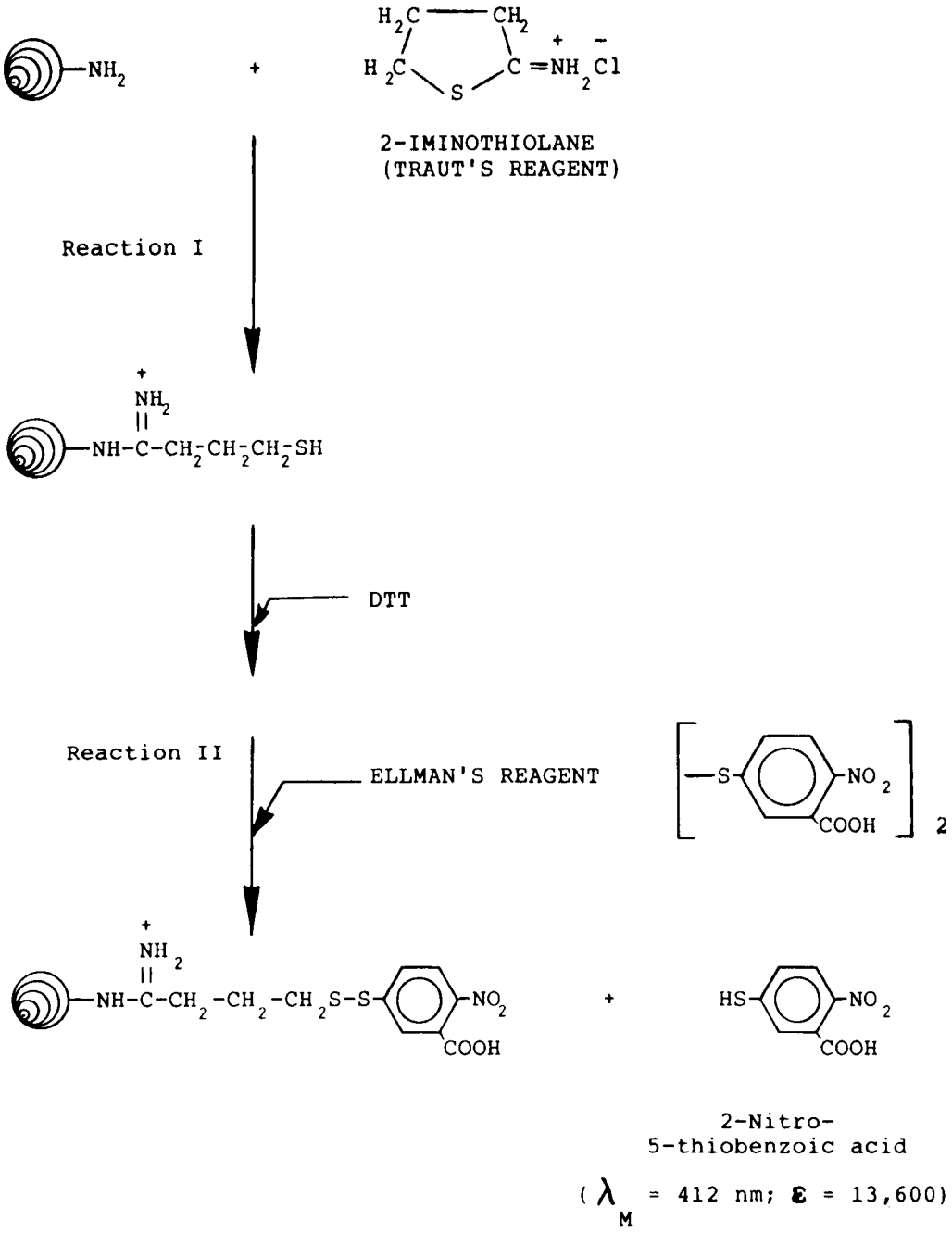

Fig. 1. Reactions involved in the colorimetric determination of the reactive amino groups of a solid support.

gent. After removing the excess, unreacted ITL, the solid support was briefly treated with DTT to ensure that the covalently bound sulfhydryl groups remained in the reduced state, not as disulfide or other oxidized forms that could interfere with the subsequent analysis. Following thorough washing steps, the solid-phase sulfhydryl groups were quantified by using Ellman's DTNB reagent. The DTNB reacts with sulfhydryl groups at pH 8 to produce 1 mol of 2-nitro-5-thiobenzoic acid per mole of sulfhydryl group (Reaction 2, Fig. 1).

MATERIALS AND METHODS

Chemicals

The ITL, DTNB, and DTT were from Sigma Chemical Co. (St. Louis, MO); Sepharose CL-4B was from Pharmacia Fine Chemicals (Piscataway, NJ); Fractogel TSK HW 75(F) was from EM Science (Cherry Hill, NJ): Trisacryl GF 2000 gel was from LKB (Gaithersburg, MD); Affi-Gel 102 was from Bio-Rad Laboratories (Richmond, CA); and Amino-Terminal Avid-Gel was from BioProbe International Inc. (Tustin, CA). All solvents were of analytical grade.

Reagents

ITL Solution (40 mM)

The ITL (27.5 mg) and 4-dimethyl-aminopyridine (24.4 mg) were dissolved in 5 mL of ethyl alcohol.

DTNB Solution (25 mM)

The DTNB (396.5 mg) was dissolved in 40 mL 0.1*M* sodium phosphate buffer containing 0.5*M* NaCl and 10 m*M* EDTA. The pH was adjusted to 8.

Procedures for Measuring the Reactive Amino Groups of a Solid Support

For Dry Solid Supports

Beads or gel particles (50 mg), were suspended in 0.5 mL ethanol contained in a 12 × 75 mm glass test tube. To this suspension was added 2.5 mL ITL solution. The test tube was stoppered and gently tumbled at room temperature (22° C) for 30 min. Then the tube was centrifuged at 2000 rpm for 1 min, and the supernatant was removed with a Pasteur pipet. The solid supports were washed successively with 2 × 4 mL ethanol; 2 × 4 mL 10 m*M* ethylene diaminotetraacetic acid (EDTA) containing 10 m*M* DTT; 2 × 4 mL 1*M* NaCl in 10 m*M* EDTA; and 2 × 4 mL 0.1*M* sodium phosphate containing 1 m*M* EDTA, pH 8. Each washing step consisted of adding a washing solution, mixing on a Vortex for 20 s,

centrifuging at 2000 rpm for 1 min, and then decanting the supernatant. To the washed solid supports were added 4 mL DTNB solution. The tube was then stoppered and tumbled at room temperature for 30 min. After centrifugation, an aliquot of the supernatant was removed and diluted 20× with 0.1*M* sodium phosphate, pH 8, containing 10 m*M* EDTA and 0.5*M* NaCl, and the absorbance of the diluted supernatant was read in a spectrophotometer at 412 nm using a similarly diluted DTNB solution as the blank. The molar extinction of 5-thio-2-nitrobenzoic acid at 412 nm is 13,600/*M* (*7*).

For Wet Solid Supports

The wet gel was first washed twice with 5× its packed volume of 0.05*M* sodium bicarbonate, pH 8.5. To 0.5 mL of packed washed gel were added 1.5 mL of the above bicarbonate buffer and 2.5 mL ITL solution. The gel suspension was immediately mixed well by using a Vortex mixer and then tumbled at room temperature for 30 min. After centrifugation and removal of the supernatant, another 1.5 mL bicarbonate buffer and 2.5 mL ITL solution were added to the gel. The gel suspension was mixed and tumbled for another 30 min. Then the tube was centrifuged at 2000 rpm for 1 min, and the supernatant was removed with a Pasteur pipet. The solid supports were washed successively with 2 × 4 mL ethanol; 2 × 4 mL 10 m*M* EDTA, containing 20 m*M* DTT; 2 × 4 mL 1*M* NaCl in 10 m*M* EDTA; and 2 × 4 mL 0.1*M* sodium phosphate, pH 8, containing 10 m*M* EDTA. Each washing step consisted of adding a washing solution, mixing on a Vortex for 20 s, centrifuging at 2000 rpm for 1 min, and then decanting the supernatant. To the washed solid supports were added 4 mL DTNB solution. The tube was then stoppered and tumbled at room temperature for 30 min. After centrifugation, an aliquot of the supernatant was removed and diluted 20× with 0.1*M* sodium phosphate, pH 8, containing 10 m*M* EDTA and 0.5*M* NaCl, and the absorbance of the diluted supernatant was read in a spectrophotometer, as described above.

RESULTS AND DISCUSSION

A convenient colorimetric method for the specific quantification of solid-supported amino groups has been developed. It is based on reacting the amine-carrying support with an amino-specific reagent ITL and, subsequently, with a thiol-specific reagent DTNB. The reaction of ITL with amino groups results in the formation of positively charged amidine linkages and, concomitantly, the introduction of sulfhydryl groups through the breaking of the thiolane ring (*6–8*). The solid-supported sulfhydryls are then titrated by using Ellman's reagent. The overall reaction sequence and experimental steps of the method are schematically summarized in Fig. 1.

Using this method I have quantified the reactive amino groups of five different matrices (Table 1). As expected, the three gels, Trisacryl GF 2000, Fractogel TSK HW75(F), and Sepharose CL-4B, which did not contain amino group, showed no absorbance at 412 nm and therefore indicated the absence of an amino group. However, when the same gels were subjected to amino group analysis by a less specific method, such as one that used an acylating reagent (*5*), they all gave a low but measurable absorbance that indicated the presence of amino groups at low concentrations. Such false-positive results are most likely the result of the lack of a reaction specificity of the acylating reagent toward amino groups. Acylating reagents are known to react with, in addition to amino groups, sulfhydryl, hydroxyl, imidazole, and phenolic groups (*10*). Two other gels that contained significant amount of amino groups, Affi-Gel 102 and Amino Terminal Avid-Gel, when tested with the present method, showed the presence of, respectively, 10.7 and 12.6/μmol amino group/mL gel swollen in aqueous solution. These values are slightly lower than those reported previously, which were, respectively, 13.3 and 13.8 μmol/mL gel. There are several possible explanations for such a slight discrepancy. First, the present method measures only those amino groups that are accessible and reactive with ITL. It does not measure amino groups that are buried, sterically hindered, or otherwise unable to react with ITL, and, yet, these amino groups may still be measurable by, for example, titration with a mineral acid. Second, the measurement of the volume of solid support with a high degree of exactness is inherently difficult. The third possible source of the discrepancy is the different degree of swelling of different gels in a solvent. Such a heterogeneity in the swelling properties of gels may result in different amounts of gel, in terms of dry weight of the gel, being packed in a unit volume that is the basis of all comparisons. The fourth possible factor is the possibility of air oxidation of the sulfhydryl groups (—SH) to disulfide bonds (—S—S—). Furthermore, because of the specificity of ITL in its reactivity toward an amino group, the present method is less likely to give false–positive results.

TABLE 1
Colorimetric Determination of the Reactive Amino Group Content of Solid Supports

Solid support	Absorbance at 412 nm	Amino group, μmol/mL gel
Trisacryl GF 2000	0.0	0
Fractogel TSK HW 75F	0.0	0
Sepharose CL-4B	0.0	0
Aminoalkyl gel (Affi-Gel 102)	0.91	10.7
Amino-terminal Avid-Gel	0.43	12.6

CONCLUSION

In conclusion, I have described a new, rapid, and convenient colorimetric method that specifically quantifies the reactive amino groups of a solid matrix. The method uses two commercially available reagents; (1) Traut's reagent, 2-iminothiolane (ITL), which reacts specifically with an amino group, and (2) Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which is a thiol-specific reagent.

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