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Synthesis and Properties of Vinyl Monomer/Enzyme Conjugates

Conjugation of L-Asparaginase with *N*-Succinimidyl Acrylate

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ABSTRACT

Monomer conjugation of an enzyme followed by copolymerization with free monomer is a useful method of enzyme immobilization. L-asparaginase was conjugated with *N*-succinimidyl acrylate. Analysis of the conjugated enzyme via isoelectric focusing showed that a molar ratio of 9.5 free monomers per enzyme was needed during the conjunction for each vinyl group bound. Only 3% of the enzyme activity was lost per vinyl group added, and conjugation of an average of four monomers per enzyme thermally destabilized the enzyme only at temperatures above 50°C. Activity of the enzyme at physiological temperatures was relatively unaffected.

Index Entries: Enzyme immobilization; vinyl monomer-enzyme conjugation; L-asparaginase; *N*-succinimidyl acrylate; enzyme-polymer conjugates; activation energy.

INTRODUCTION

Immobilized enzymes have many applications for use in both medicine and industry (*1–3*). In this paper we will discuss an infrequently used immobilization technique, which will be referred to as "monomer

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conjugation plus copolymerization." In this technique, displayed schematically in Fig. 1, an enzyme is conjugated with a vinyl group and is then copolymerized with free monomer. This results in a copolymer con-

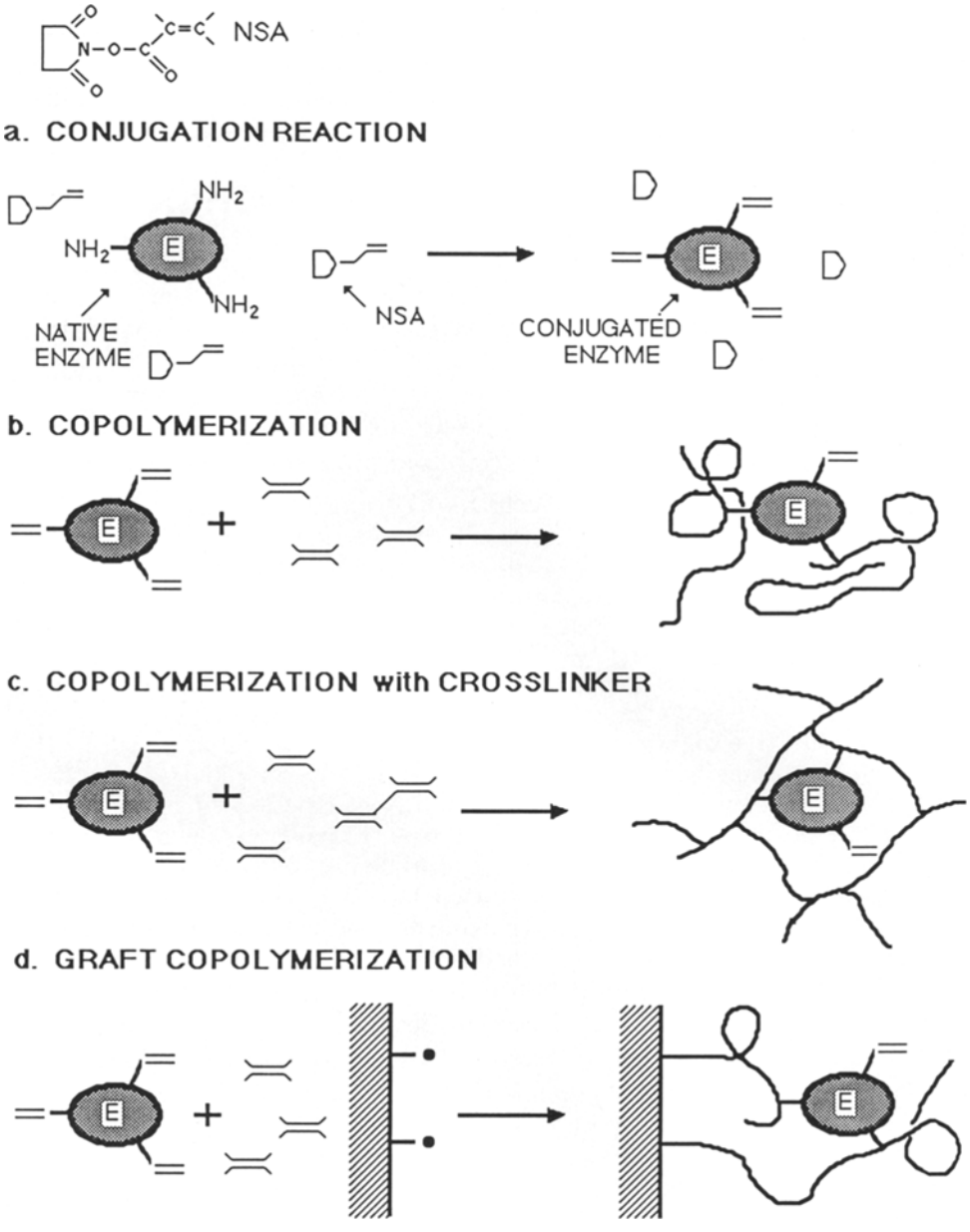

Fig. 1. Enzyme conjugation and uses of conjugation product: (a) Conjugation reaction; (b) copolymerization of conjugated enzyme with comonomer; (c) Copolymerization of conjugated enzyme with comonomer and crosslinker producing an enzyme-containing gel; (d) copolymerization of conjugated enzyme with comonomer via initiation by surface radicals producing an enzyme-containing graft.

jugated with an enzyme, thus immobilizing it. If crosslinker is included in the polymerization mixture, a gel containing an immobilized enzyme will be formed (*4,5*). In addition, surface radicals generated on a polymer support can be used to form a surface graft copolymer containing immobilized enzyme (*6,7*). This method has significant advantages as an enzyme immobilization system because it allows the enzyme to be separated from the support on a flexible polymer chain. In other immobilization methods, such as support activation, the enzyme is located close to the support, allowing interactions with the matrix and causing a reduction in mobility. Consequently, immobilization via copolymerization allows the enzyme to have more freedom and more accessibility to the substrate. This method also has not only been used for the immobilization of enzymes (*4,7,8*), but also to immobilize other molecules, such as monoclonal antibodies (*9*), carbohydrate ligands (*10*), boric acid derivatives (*11*), and ATP (*12*).

N-succinimidyl acrylate (NSA) has previously been used in enzyme immobilization as a conjugating monomer (*4*) and also as a comonomer, which, when copolymerized with other monomers such as acrylamide, forms a copolymer that can be reacted with solutions of enzymes, antibodies, or other biomolecules for subsequent immobilization of those compounds (*13–16*). Figure 2 shows the reaction of NSA with a lysine group on an enzyme; the succinimidyl group is displaced by the lysine amino group, resulting in addition of a vinyl group to the enzyme and production of *N*-hydroxy succinimide. For our conjugation system, NSA is used to conjugate vinyl groups to L-asparaginase for further application in an immobilization system. L-asparaginase was chosen as the model enzyme for this system because of its clinical relevance: It is currently being used as therapeutic treatment for childhood acute leukemia (*17*). This enzyme was also chosen because of its relatively simple activity assay and its isoelectric properties.

In this study, both the conjugation reaction and the resulting conjugated enzyme were characterized. The effectiveness of the conjugation reaction was determined by isoelectric focusing. Since NSA reacts with the ϵ-amino groups on lysine residues of the protein (*18*), the net charge of the protein will change and can be detected as a change in the enzyme's isoelectric point (p*I*). The conjugated enzyme was also tested for changes in activity, heat stability, and temperature-activity behavior. The results of this study showed that moderate levels of conjugation produced only small effects on these properties; therefore, this method is especially useful as a first step in an immobilization technique.

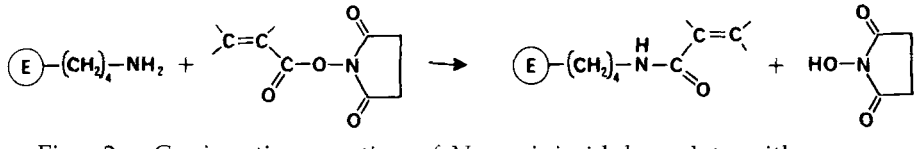

Fig. 2. Conjugation reaction of *N*-succinimidyl acrylate with an enzyme lysine group.

MATERIALS AND METHODS

N-hydroxysuccinimide and *Tris*(hydroxymethyl)aminomethane (*Tris*), L-asparagine, trichloroacetic acid (TCA), and Sigma Ammonia Color Reagent (Nessler Reagent) were obtained from Sigma. Acryloyl chloride and 2,6-di-tert-butyl-4-methyl-phenol (gold label) were purchased from Aldrich. L-asparaginase (ELSPAR®) was purchased from Merck, Sharp, and Dohme. PD-10 Sephadex gel columns were purchased from Pharmacia, and triethylamine was obtained from J. T. Baker Chemical. All other chemicals used were reagent grade.

CONJUGATION

Synthesis of NSA

The active ester of *N*-hydroxysuccinimide, NSA, was synthesized from *N*-hydroxysuccinimide and acryloyl chloride following the method of Adalsteinsson et al. (*15*). The total yield recovered was 69%.

Conjugation Reaction

L-asparaginase was dissolved in 0.29*M* carbonate buffer, 1*M* NaCl pH 9.3, at a concentration of no more than 10 mg protein/mL. The absorbance of the enzyme solution was measured at 278 nm using a Bausch and Lomb Spectronic® 1001. The extinction coefficient for L-asparaginase, ϵ_{278}, is 0.71 [(Abs/mg/mL)] (*19*).

The enzyme solution was equilibrated for 5 min at 37°C. A 5 mg/mL solution of NSA in deionized water was prepared immediately prior to addition, and pipetted into the enzyme solution in the amount needed to accomplish the desired NSA:enzyme ratio. The reaction was terminated after 60 min by addition of the enzyme solution to a PD-10 Sephadex column pre-equilibrated with at least 60 mL of 0.1*M* Tris buffer, pH 8.6. The columns were eluted by adding 0.5 or 0.25 mL aliquots of Tris buffer and collecting the resulting fractions. The fractions with detectable enzyme concentration were then pooled and used in further experiments. Two sets of conjugation reactions were performed. In one, the NSA:enzyme ratios were 8 (8×) and 16 (16×), and in another they were 16×, 32×, and 64× (each along with the native enzyme).

Determination of Extent of Conjugation

Two isoelectric focusing gels were carried out. After staining, the gels were scanned using a scanning densitometer interfaced with a Hewlett Packard computer. The peaks scanned by the densitometer were printed out by the computer and integrated to obtain the area under each peak.

The extent of conjugation of the enzyme from these experiments was calculated by taking the number average of the peaks, using the

peak areas, according to Eq. 1. Peak numbers were assigned relative to the position of the native enzyme peaks. Subtracting the unconjugated from the conjugated enzyme values gives the average number of monomers added per enzyme (Eq. 2).

$$\overline{C}_N = \frac{\sum_{i=p_i}^{p_f} pA_p}{\sum_{i=p_i}^{p_f} A_p} \quad (1)$$

$$N_{conj} = \overline{C}_N - \overline{U}_N \quad (2)$$

where

N_{conj} = number of monomers attached per enzyme;
$\overline{C}_N$ = number average of conjugated enzyme scan;
$\overline{U}_N$ = number average of unconjugated enzyme scan;
p = peak number;
p_i = peak number of initial conjugated enzyme peak;
p_f = peak number of final conjugated enzyme peak; and
A_p = area of peak.

L-Asparaginase Activity Assay

Ammonia produced from the hydrolysis of L-asparagine was measured using Nessler's reagent as follows (*19*). A 0.6-mL volume of 17 m*M* L-asparagine in 50 m*M* Tris buffer, pH 8.6, was equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.1 mL of L-asparaginase (20 μg/mL in 0.1*M* Tris pH 8.6), and, after 10 min at 37°C, was terminated with 50 μL of 1.5*M* TCA. The blank for the assay was prepared by adding TCA to the asparagine/buffer solution before the addition of the enzyme.

A 0.2-mL aliquot of reaction solution was added to 3.8 mL of deionized water, then combined with 0.5 mL Nessler reagent. After 10 min, the absorbance at 420 nm was measured against the blank. The specific activity of the enzyme in International Units (IU) was calculated by constructing a standard curve, using ammonium sulfate as the source of ammonia in the Nessler assay.

Thermal Stability

To determine the effect of conjugation on the thermal stability of the enzyme, enzyme conjugated with a 40:1 ratio of monomers to enzyme in the reaction solution was tested for activity retention after incubation at different temperatures. Solutions of 0.02 mg/mL L-asparaginase in Tris

buffer were incubated at 37, 50, and 70°C. Aliquots were removed at intervals and assayed for activity using the Nessler activity assay.

Activation Energy

The temperature dependence of soluble enzyme activity was determined by testing the enzyme's activity at varying temperatures. The reaction temperature was varied in 4° increments, between 16 and 40°C, using a controlled temperature shaker bath. The enzyme reaction was performed in the manner outlined in the previous section, with the substrate/buffer mixture being allowed to equilibrate at the reaction temperature for at least 10 min before reaction.

RESULTS AND DISCUSSION

Synthesis of NSA

A nuclear magnetic resonance (NMR) spectrum was obtained on the product using deuterated chloroform ($CDCl_3$) as the solvent and tetramethylsilane (TMS) as the standards. The spectrum is shown in Fig. 3. The set of peaks at the left of the spectra, labeled "a," represents the vinylic hydrogens, and the peak to the right, labeled "b," represents the hydrogens in the succinimidyl group. The integration trace, representing the area under the peaks and, therefore, the relative amount of that type of hydrogen present, is also shown on the scan (labeled "c"). The ratio of the area under the two peaks (3.86:3) concurred with the expected ratio of vinylic to succinimidyl hydrogens (4:3). No impurity peaks are visible. This result showed that our synthesis produced relatively pure NSA.

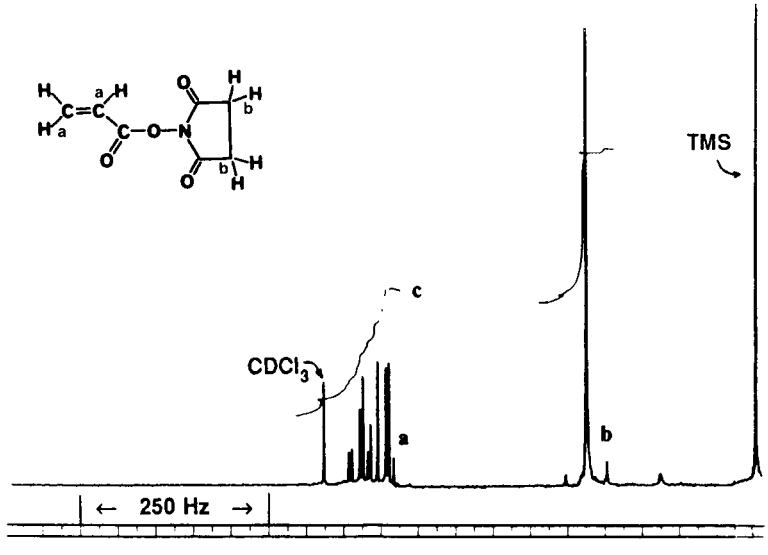

Fig. 3. NMR scan of *N*-succinimidyl acrylate: a = vinylic H's; b = succinimidyl H's; and c = integration trace.

Conjugation Reaction

A stained IEF gel from three conjugation reactions is shown in Fig. 4. In the first column of the figure, the main peak of the native enzyme can be clearly seen along with the two subpeaks. The second three lanes represent the products of conjugation reactions, with initial monomer-to-enzyme ratios of 16×, 32×, and 64×, respectively. The patterns for the conjugated enzymes show that the reaction produces a distribution of conjugation levels. The scan from a similar gel performed on an 8× reaction product is displayed in Fig. 5, along with a control gel. Calculated average conjugation levels for various gels are plotted in Fig. 6.

This graph shows that there is a linear relationship between the NSA:enzyme ratio in solution and the extent of conjugation (within the range of NSA:enzyme ratios studied). According to the amino acid sequence of the enzyme, there are 20 lysine residues in each subunit (*20*). Not all of these lysines will be available for bonding because some will be located on the inside of the protein. If the ratio of monomer-to-enzyme is increased, the curve shown in Fig. 6 would be expected to level out as all of the available lysines are reacted.

From the slope of the line it was found that an NSA:enzyme ratio in solution of 9.5:1 produced an average of one monomer bound per enzyme.

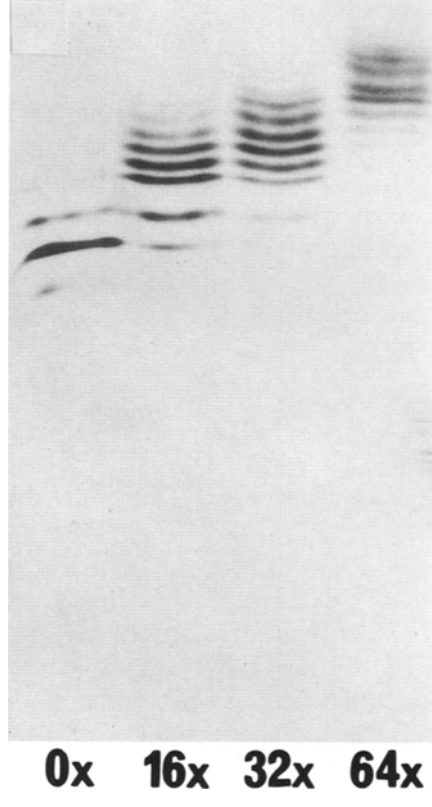

Fig. 4. Isoelectric focusing gel from three enzyme conjugation reactions.

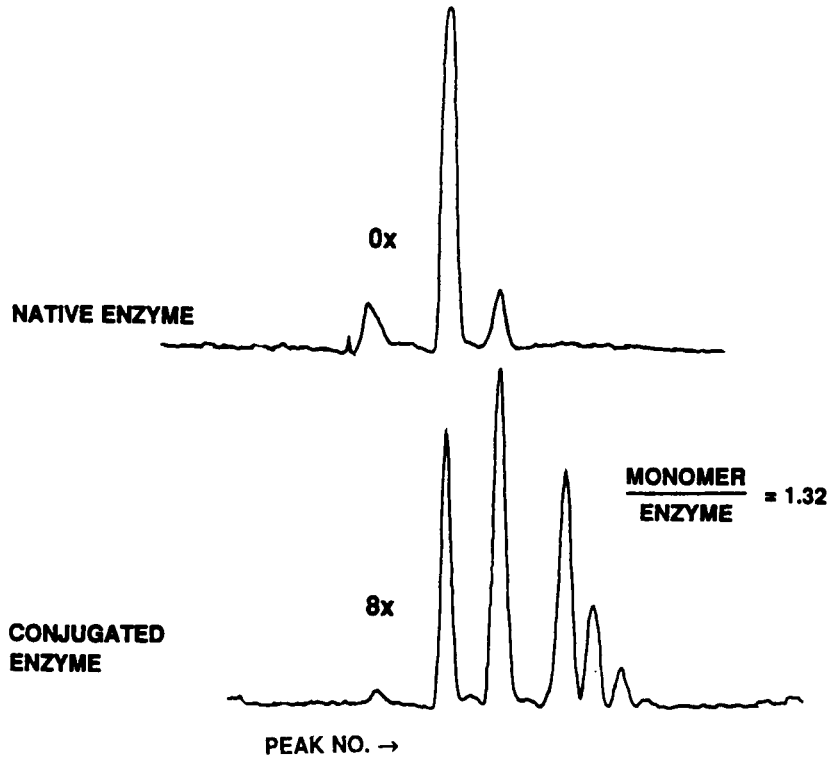

Fig. 5. Densitometry scans showing native and conjugated enzymes.

Our reaction may be made more efficient if another solvent is used for the NSA instead of water, since there is a tendency for NSA to hydrolyze into NHS and acrylic acid in aqueous solution (*14*). However, since many organic solvents would denature the enzyme, we continued to use an aqueous system.

Effect of Conjugation on Enzyme Activity

The effect of the conjugation level on the enzyme activity at 37°C was studied by measuring the activity of conjugated enzymes for which the number of attached monomers had been determined (estimated from Fig. 6). The monomer/enzyme ratios tested were 16×, 32×, and 64×. The results of the activity experiments are shown in Fig. 7.

These data show that a relatively high level of enzyme activity is conserved through the conjugation reaction: The addition of as many as eight vinyl groups leaves 80% of the enzyme activity intact. It can also be seen that there is a linear decrease in activity with an increase in the number of vinyl groups that have been conjugated to the enzyme. This drop in activity could be caused by several factors. The most probable reason for this drop is that the monomer has reacted with a lysine that is part of the active site of the enzyme. A reaction of this type would result in inactivation of a fraction of the enzymes in solution, producing a frac-

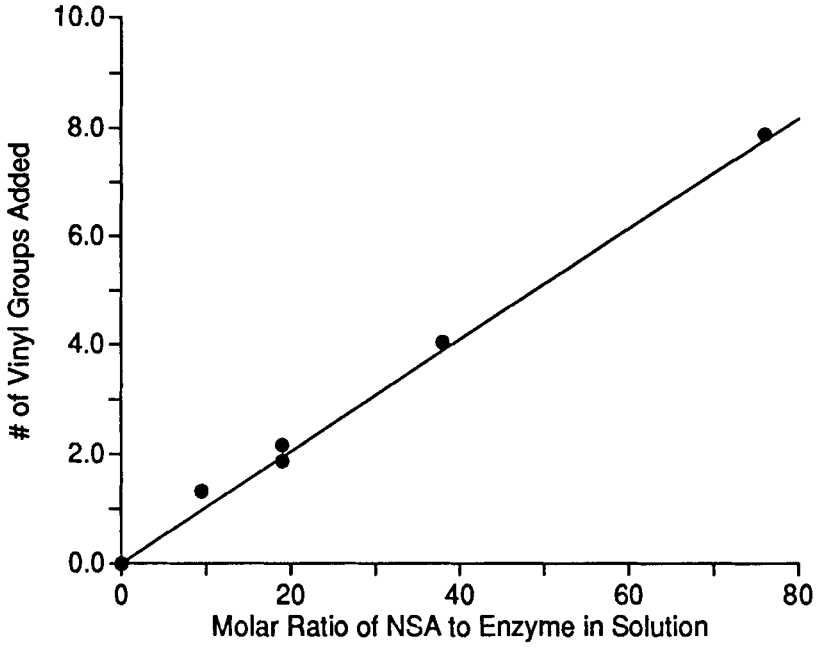

Fig. 6. Efficiency of NSA–L-asparaginase conjugation; reaction temperature = 37°C, reaction time = 1 h, pH = 9.3.

tional loss in total solution activity. In addition, the monomer could be bound in such a way that it sterically hinders access of the substrate to the active site. Further, monomer conjugation could change the protein's conformation, which can influence substrate binding (*21*). These types of reactions would lower the solution activity by lowering the specific activity of the enzyme. The probability that one of these unfavorable reactions will take place increases as the number of vinyl groups per enzyme increases.

Thermal Stability

The effect of incubation at elevated temperatures on the activities of the native and conjugated enzymes (conjugation level = 4 monomers/enzyme) is shown in Figs. 8 and 9. Comparing the two graphs, a striking difference can be seen between the behavior of the conjugated and unconjugated enzyme at 50°C. Although there is no significant difference between the activities at 50 and 37°C for the native enzyme, the conjugated enzyme shows a marked drop in activity after incubation at 50°C. It is also interesting to note that the activity appears to level out after about 100 min at 50°C. In addition, this decreased activity was shown to be an irreversible inactivation.

These results suggest that the conjugation of vinyl groups onto an enzyme destabilizes its tertiary structure, lowering the energy threshold needed for it to make a conformational change to an inactive or less-

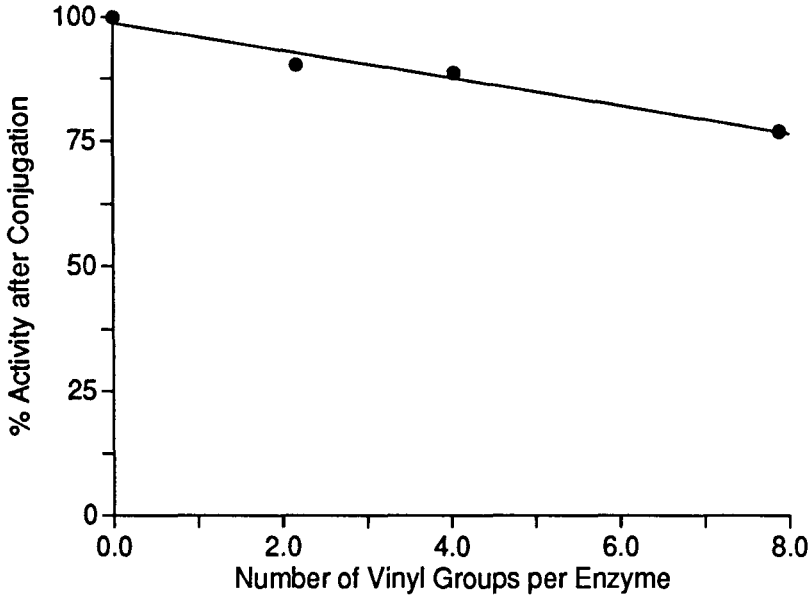

Fig. 7. Effect of conjugation on enzyme activity.

active form. The plateau in the conjugated enzyme activity at 50°C can suggest two deactivation mechanisms. One hypothesis is that, upon heating, the enzyme converts into a less active conformation than that of the native enzyme. Another explanation would be that a fraction of the enzyme molecules in solution had vinyl groups conjugated at certain sites on the enzyme that would cause them to denature upon heating.

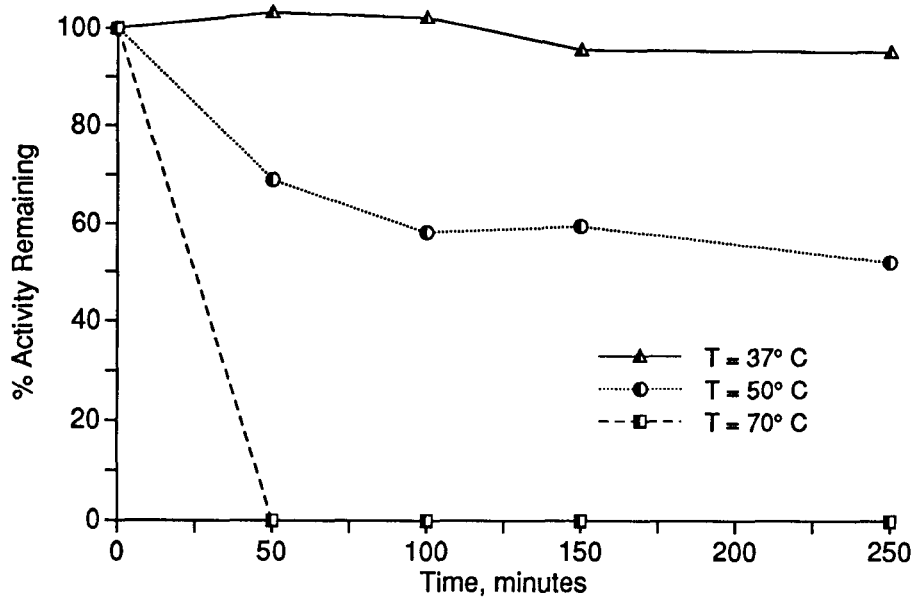

Fig. 8. Heat stability of conjugated enzyme; conjugation ratio = 4 monomers/enzyme.

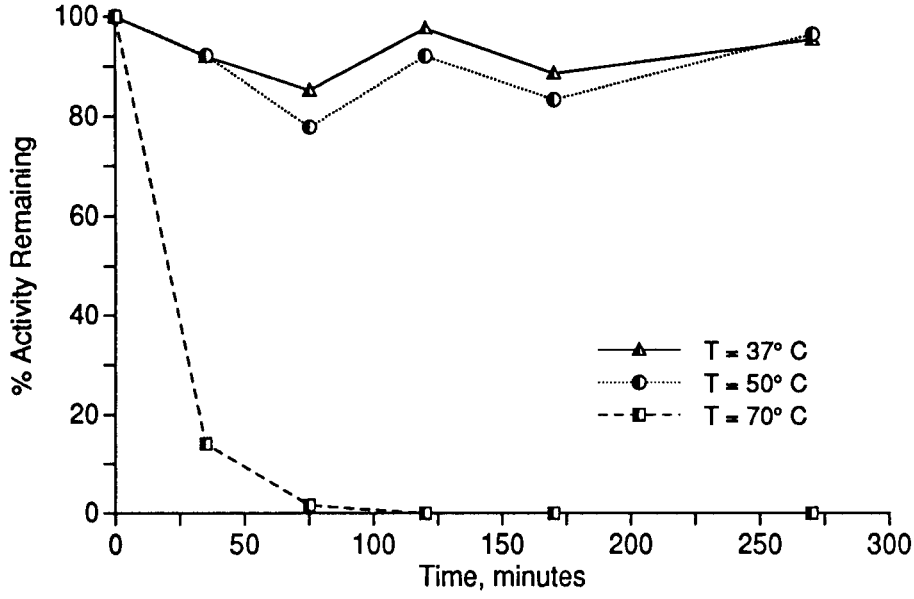

Fig. 9. Heat stability of native enzyme.

This effect can be referred to as an "all-or-none"inactivation mechanism, in which the rest of the enzyme molecules in solution would retain their original activity, but the specific activity of the solution would be decreased. It is also conceivable that both of these mechanisms could be working together.

Activation energy E_a

The activation energies of the native and conjugated enzymes were determined from the activity vs temperature data using the method of Arrhenius. Temperatures for these studies were chosen to be within the range for which the enzyme could be incubated without significant deactivation ($T \leq 40°C$). The Arrhenius plot obtained for native and conjugated enzymes is shown in Fig. 10. The activation energies were found from the slopes of the plots, which were determined by a least-squares fit. It can be seen that there are two distinct regions in each plot, with a discontinuity at ca. 32°C.

The observation of two significantly different slopes on these Arrhenius plots agrees with the results of Milman, who in 1979 reported a break in the Arrhenius plot for L-asparaginase at 30°C, close to where we observe it (22). According to Talsky, as many as 29 enzymes have been observed to exhibit anomalies in their Arrhenius plots (23). He investigated these inconsistencies by performing temperature/activity measurements using small temperature increments (1–2°). He found that not only do enzymes exhibit temperature anomalies, but sometimes the entire Arrhenius plot is wave-like, containing no real straight-line regions. He concluded that the anomalous temperature dependence of the reaction

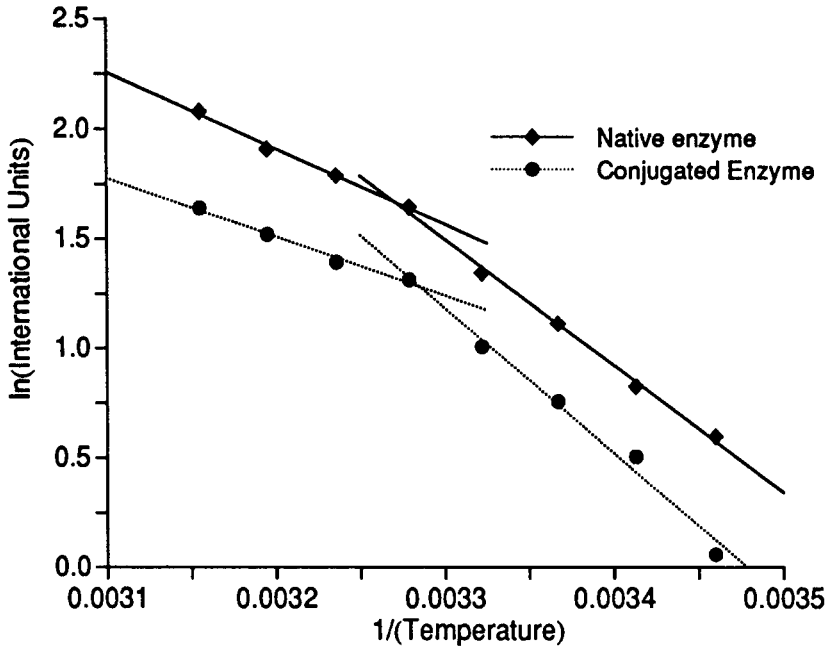

Fig. 10. Arrhenius plot for native and conjugated L-asparaginase; conjugation ratio = 10 monomers/enzyme.

was caused by minute conformational changes in the enzyme that become particularly important when they affect the enzyme–substrate complex. Other conditions that affect this phenomenon are pH and substrate concentration.

Considering Talsky's work, an explanation for the temperature behavior of asparaginase is that it undergoes some type of conformational alteration between 28 and 32°C. Table 1 shows the values of E_a that were determined for both the upper and lower slopes seen in Fig. 10. The table also shows the ratio of the upper-to-lower slopes for both enzymes.

The value obtained by Milman for the E_a of native L-asparaginase below 30°C was 13.1 kcal/mol. Our value (11.5 kcal/mol) is of the same order of magnitude, but is actually closer to the value of 11.2 kcal/mol obtained by Charlson, who assumed only one slope (*24*). The data also show that there is a significant difference between the activation energies of the two enzyme types for both the upper and lower temperature ranges. The upper slope of the conjugated enzyme curve is less steep than the native enzyme, whereas the lower slope is more steep, causing the ratio of the two slopes to be much smaller than for the native enzyme. These data show that vinyl conjugation has a measurable effect on the activation energy of the enzyme. The conformational changes suggested by Talsky would explain these results. These changes appear to assist the enzyme reaction above 32°C and hinder it below that value. Since physiological temperature is 37°C, the enzyme is still very effective for clinical use.

TABLE 1
Effect of Conjugation on the Calculated E_a Above and Below Transition on Arrhenius Plot

	Native enzyme	Conjugated enzyme
E_a, kcal/mol, $T \leq 32°C$	11.5 ± .4	13.2 ± .8
E_a, kcal/mol, $T \geq 32°C$	6.8 ± .3	5.3 ± .4
Ratio #2/#1	0.60 ± .04	0.40 ± .04

CONCLUSIONS

This research has shown that vinyl groups can be conjugated to proteins via reaction with NSA. The conjugation method presented here does not significantly decrease the activity of the enzyme; only 3% of the activity is lost with each monomer group added. Conjugation of four monomers per enzyme thermally destabilizes the enzyme only at temperatures above 50°C; activity of the enzyme at physiological temperatures is relatively unaffected. In addition, it was found that L-asparaginase exhibits a temperature anomaly in its Arrhenius plot at $T \cong 32°C$, and that the conjugation reaction lowers the activation energy at temperatures above this value and raises the activation energy at temperatures below it. In conclusion, this conjugation method produces a vinylized enzyme that can subsequently be immobilized by copolymerization with a free monomer. Research is currently being conducted on immobilized enzymes utilizing this method.

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