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Enhancement by *N*-Hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions[1]

JAMES V. STAROS, RICK W. WRIGHT,[2] AND DEBORAH M. SWINGLE

Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

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Water-soluble carbodiimides are frequently employed in coupling or conjugation reactions, e.g., to link a peptide immunogen to a carrier protein. However, their utility is limited by low coupling yields obtained under some conditions. We have found that addition of *N*-hydroxysulfosuccinimide to such reactions can greatly enhance the yields obtained.

KEY WORDS: *N*-hydroxysulfosuccinimide; carbodiimide; coupling; peptides; proteins; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

Carbodiimides catalyze the formation of amide bonds between carboxylic acids and amines by activating the carboxylate to form an *O*-acylurea (1,2). This intermediate either can be attacked by the amine directly to form the amide (3) or it can be attacked by a second carboxylate to give the anhydride which can then be attacked by the amine, giving the amide and regenerating one of the carboxylates (1,3). When this reaction is carried out in an aqueous milieu with a water-soluble carbodiimide, the activated species are subject to hydrolysis, and this hydrolysis can severely limit the overall yields obtained. We hypothesized that if we could "rescue" the activated species by formation of a more hydrolysis-resistant active intermediate, the yield of the reaction could be greatly enhanced.

N-Hydroxysulfosuccinimide [$HOSu(SO_3)$][3] esters (4) are hydrophilic active esters that hydrolyze very slowly compared with their rates of reaction with primary amino groups (5). A number of monofunctional (5–7) and bifunctional (4,8–11) $HOSu(SO_3)$ active esters have been reported and have been successfully employed as protein modification reagents. Here we describe the use of *N*-hydroxysulfosuccinimide to enhance the yields of water-soluble carbodiimide-mediated coupling reactions, presumably by the formation of the $HOSu(SO_3)$ active esters *in situ*.

EXPERIMENTAL PROCEDURES

$HOSu(SO_3)$ sodium salt was synthesized as previously described (4).[4] 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was from Pierce Chemical Company, [U-^{14}C]glycine was from ICN Biomedicals, Inc., and hemocyanin from *Megathura crenulata* (keyhole limpet) in a slurry in 65% $(NH_4)_2SO_4$ was from Calbiochem Biochemicals, Behring Diagnostics. All other reagents were of the highest grade available. Water was deionized and then distilled in glass.

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[2] Present address: University of Missouri Medical School, Columbia, Mo. 65201.

[3] Abbreviations used: $HOSu(SO_3)$, *N*-hydroxysulfosuccinimide; $HOSu(SO_3)Na$, $HOSu(SO_3)$ sodium salt; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

[4] $HOSu(SO_3)$ sodium salt is available commercially from Pierce Chemical Co., P.O. Box 117, Rockford, Ill. 61105, and from Fluka Chemical Corp., 255 Oser Avenue, Hauppage, N.Y. 11788.

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The hemocyanin suspension was dialyzed at 3°C against 5 mM sodium phosphate, pH 7.4. This dialysis step was necessary to solubilize the majority of the hemocyanin. Any remaining insoluble material was removed by centrifugation at 47,800*g* for 15 min at 3°C. The hemocyanin was then dialyzed against distilled water. Protein concentrations were determined by the method of Lowry *et al.* (12).

[U-^{14}C]Glycine was received in solution in 0.01 N HCl. A stock of [^{14}C]glycine was prepared by adding an aliquot of the radioactive glycine to a solution of nonradioactive glycine in distilled water and then neutralizing the solution by addition of 1 equivalent of $NaHCO_3$ (relative to the HCl present) to give a stock concentration of 40.5 mM and a specific activity of 62 μCi/mmol.

Coupling reactions were initiated by adding EDC to a concentration of 0.1 M to an aqueous solution of hemocyanin, [^{14}C]glycine, and $HOSu(SO_3)Na$ to give a total volume of 0.30 ml. Coupling reactions were incubated overnight at room temperature. Reactions were terminated by addition of the reaction mixture to 5 ml of ice-cold 10% trichloroacetic acid. After 15 min on ice, the samples were collected by filtration on Whatman GFB filters. The filters were then washed four times with ~3 ml of ice-cold 10% trichloroacetic acid, followed by one wash of ~2 ml of 95% ethanol. After the filters were allowed to air-dry, they were transferred to scintillation vials and 10 ml of ACS cocktail (Amersham Corp.) was added. After incubation for 16–100 h at room temperature, the samples were counted in a Beckman LS7500 liquid scintillation counter. Samples were corrected for background by subtracting the radioactivity in a parallel sample containing hemocyanin and [^{14}C]glycine but lacking EDC and $HOSu(SO_3)$. All samples were prepared in triplicate.

RESULTS AND DISCUSSION

As a model system for assessing the effect of $HOSu(SO_3)$ on water-soluble carbodiimide-mediated coupling reactions, we examined the coupling of [^{14}C]glycine to keyhole limpet hemocyanin under conditions comparable to those used to couple peptide immunogens to carrier proteins. The use of glycine as a "model peptide" probably results in yields of covalent incorporation that are near the lower limit of those that would be observed with peptides, as the amino acid has only one amino group and one carboxylate group that can participate in the conjugation reaction. The effect of $HOSu(SO_3)$ on yields of incorporation in this system is dramatic (Fig. 1). At 5 mM $HOSu(SO_3)$, the covalent incorporation of glycine (at 3.1 mM) is ~15-fold greater than it is in the absence of $HOSu(SO_3)$. Comparable enhancements in yield are observed over a 10-

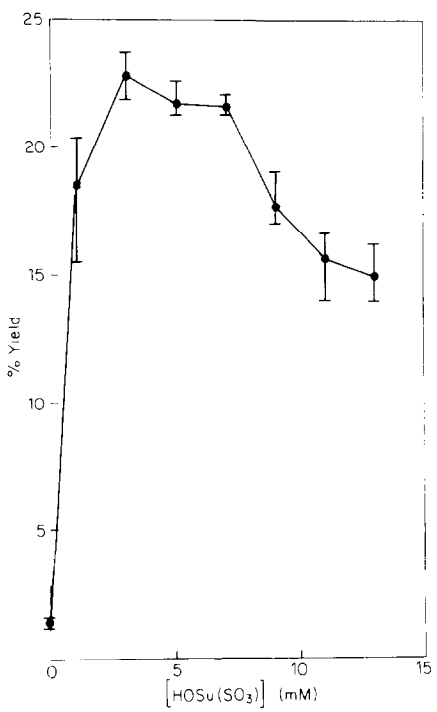

FIG. 1. Effect of *N*-hydroxysulfosuccinimide concentration on coupling yield. The reaction mixtures contained 7.0 mg/ml keyhole limpet hemocyanin, 3.1 mM [^{14}C]glycine, 0.10 M EDC, and variable concentrations of *N*-hydroxysulfosuccinimide [$HOSu(SO_3)$]. "% Yield" refers to the percentage of total glycine added that was precipitated with hemocyanin, corrected for background. See Experimental Procedures for details. Mean values and ranges for triplicate samples are plotted.

TABLE 1

EFFECT OF GLYCINE CONCENTRATION ON COUPLING YIELD[a]

[Glycine] (mM)	[HOSu(SO_3)] (mM)	Yield (%)
1	0	0.9
1	5	20.6
3	0	1.4
3	5	20.8
10	0	0.9
10	5	16.1

[a] Experimental conditions were as described in Fig. 1, except that the concentration of [^{14}C]glycine was varied as indicated.

fold range of glycine concentration (Table 1). Experiments with bovine serum albumin in place of hemocyanin gave similar results, though the absolute yields obtained in both the presence and absence of HOSu(SO_3) are somewhat higher: for bovine serum albumin (7.0 mg/ml), [^{14}C]glycine (3.1 mM), and EDC (0.1 M), 1.9% yield; with the addition of 6.7 mM HOSu(SO_3)Na, 37.6% yield.

Results presented here suggest that addition of HOSu(SO_3) to EDC-mediated peptide–protein coupling reactions should significantly improve coupling yields. The addition of HOSu(SO_3) to other water-soluble carbodiimide-mediated coupling reactions, such as the coupling of protein ligands to chromatographic supports for affinity chromatography, is likely to result in similar enhancements in yield.

REFERENCES

1. Khorana, H. G. (1953) *Chem. Rev.* **53,** 145–166.
2. DeTar, D. F., and Silverstein, R. (1966) *J. Amer. Chem. Soc.* **88,** 1013–1019.
3. DeTar, D. F., and Silverstein, R. (1966) *J. Amer. Chem. Soc.* **88,** 1020–1023.
4. Staros, J. V. (1982) *Biochemistry* **21,** 3950–3955.
5. Anjaneyulu, P. S. R., and Staros, J. V. (1986) Submitted.
6. Kotite, N. J., Staros, J. V., and Cunningham, L. W. (1984) *Biochemistry* **23,** 3099–3104.
7. Donovan, J. A., and Jennings, M. L. (1986) *Biochemistry* **25,** 1538–1545.
8. Denney, J. B., and Blobel, G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5286–5290.
9. Jennings, M. L., and Nicknish, J. S. (1985) *J. Biol. Chem.* **260,** 5472–5479.
10. Ludwig, F. R., and Jay, F. A. (1985) *Eur. J. Biochem.* **151,** 83–87.
11. Beth, A. H., Conturo, T. E., Venkataramu, S. D., and Staros, J. V. (1986) *Biochemistry* **25,** in press.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.