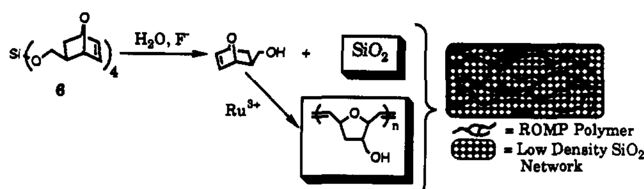
Scheme I



organic polymer. Contrast between the two phases can be best achieved by etching away (oxygen plasma)[12] the organic polymer to leave behind the textured glass surface, revealing cavities left by the polymer. Comparison of an etched SIPN composite with a transparent composite made from a preformed polymer (cellulose acetate) clearly displays a smaller domain size for SIPN composites over those materials derived from preformed polymers (Figure 1).

A significant drawback associated with the sol-gel process is removal of the cosolvent, excess water, and liberated alcohol. Xerogels routinely shrink 70-80% during the drying process,[5,13] which precludes molding processes and can introduce a high degree of stress within materials. Because of this shrinkage, gels must be dried at unreasonably slow rates to avoid cracking and deformation of the gel.[5] In an effort to overcome the problems associated with xerogel formation, we have synthesized tetraalkoxysilanes from $SiCl_4$ and the strained, cyclic alkenols **2** and **5**.[14] By employing polymerizable monomers such as monomers **2**, **3**, **4**, or **5** as the cosolvent, the tetraalkoxysilane derivative, and a stoichiometric quantity of water, all components of the composite solution contribute to either the SiO_2 network or the organic polymer. Since both the cosolvent and the liberated alcohol polymerize, gel drying is unnecessary and no gel shrinkage occurs (Scheme I).

Since the nonshrinking formulations are essentially bulk polymerizations, kinetic studies of the ROMP rates and condensation rates at high monomer concentrations were studied in order to verify simultaneous completion of both reactions. At high concentrations, the polymerization of alcohol **5** shows first-order behavior between 4.0 and 7.0 M ($k = 1.9 \times 10^{-2}\ s^{-1}$). At higher concentrations (pertinent for the bulk polymerization of **6**), significant deviation toward faster rates is observed. Consistent with these observations, the ROMP of **5** is greater than 90% complete after 1.5 min. It is most convenient to match the sol-gel rates with these ROMP rates by adjusting the concentration of the condensation catalyst (NaF). For example, in the absence of ROMP catalysts, the condensation of compound **6** with stoichiometric water can be varied from several seconds to several days by simply adjusting the NaF concentration. To match the above ROMP rates, gel times of 1.5-2.0 min at 60 °C can be achieved by using NaF solutions near 50 mM. Significant deviations from these matched rates result in systems that approach the homopolymerization limits: uncontrolled polymer precipitation when the ROMP rate is greater than the condensation rate, or brittle glasses which shrink (due to unreacted monomer evaporation) when the condensation rate is much greater than the ROMP rate. Under ideal reaction conditions, a transparent glass-polymer composite is obtained with no observable shrinkage.

Acknowledgment. This work was supported by University of California "Startup Funds", the Center for Advanced Materials, Materials and Chemical Sciences Division, Lawrence Berkeley Laboratory, and E. I. du Pont de Nemours and Company. M.W.E. gratefully acknowledges the DOED for a graduate fellowship.

(12) Etching performed with a Plasmod oxygen plasma etcher, courtesy of Dr. Donald Scales, California State Department of Public Health, Berkeley, CA.

(13) Brinker, C. J.; Scherer, G. W. *Sol-Gel Science. The Physics and Chemistry of Sol-Gel Processing*; Academic Press: New York, 1990.

(14) Ebelmen, J. J. *Justus Liebigs Ann. Chem.* **1846**, *57*, 331.

Construction of a Light-Activated Protein by Unnatural Amino Acid Mutagenesis

David Mendel, Jonathan A. Ellman, and Peter G. Schultz*

Department of Chemistry, University of California
Berkeley, California 94720
Center for Advanced Materials
Lawrence Berkeley Laboratory
One Cyclotron Road
Berkeley, California 94720

Received December 18, 1990

Low molecular weight caged substances, inactive precursor molecules that can be activated photochemically, are useful tools for biochemical studies.[1-4] Photolabile acyl enzyme adducts are also of interest in triggering enzymatic reactions.[5] We now report a general approach to the construction of *caged proteins* based on methodology that allows site-specific incorporation of unnatural amino acids into proteins.[6,7] This development should make possible a broad range of time-resolved experiments relevant to protein folding, protein-protein and protein-ligand interactions, catalytic mechanisms, and protein conformational studies. Here we describe the construction of a photoactivatable phage T4 lysozyme (T4L) containing an aspartyl β-nitrobenzyl ester (NB-Asp) in the active site.

T4L is a stable, well-characterized enzyme whose lysis of *Escherichia coli* cells provides a convenient and sensitive assay for catalytic activity.[8] Aspartic acid 20 (Asp 20) and glutamic acid 11 (Glu 11) oppose one another across the binding cleft floor and are essential for catalytic activity.[8,9] It has been proposed that Glu 11 of T4L donates a proton to the interglycosidic oxygen of $\beta(1\rightarrow4)$-linked NAM-NAG residues of the cell wall and that the incipient oxonium ion decomposes to give a truncated polysaccharide and a carbocation which is stabilized by Asp 20.[10] An aspartyl β-nitrobenzyl ester was therefore introduced at position 20 to produce a catalytically inactive (caged) lysozyme. Upon irradiation, nitrobenzyl esters are known to undergo rapid (on the order of milliseconds[11]) conversion to the free acid plus *o*-nitrosobenzaldehyde. Irradiation of the inactive, NB-Asp-containing lysozyme thus generates a fully active protein.

Because suppression of the Asp 20 amber mutant is carried out in vitro, it was necessary to verify that protein synthesized in vitro is the same as protein synthesized in vivo. T4L[12] was expressed and purified to homogeneity from the cell-free coupled transcription-translation system of Zubay[13] programmed with the

* Author to whom correspondence should be addressed.

(1) (a) Schlichting, I.; et al. *Nature* **1990**, *345*, 309. (b) Schlichting, I.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7687.

(2) (a) Blatt, M. R.; Thiel, G.; Trentham, D. R. *Nature* **1990**, *346*, 766. (b) Parker, I.; Ivorra, I. *Science* **1990**, *250*, 977. (c) Adams, S. R.; et al. *J. Am. Chem. Soc.* **1988**, *110*, 3212. (d) Adams, S. R.; Kao, J. P. Y.; Tsien, R. Y. *J. Am. Chem. Soc.* **1989**, *111*, 7957.

(3) Gilroy, S.; Read, N. D.; Trewavas, A. J. **1990**, *346*, 769.

(4) Wilcox, M.; et al. *J. Org. Chem.* **1990**, *55*, 1585.

(5) (a) Stoddard, B. L.; Bruhnke, J.; Porter, N.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 4871. (b) Stoddard, B. L.; Bruhnke, J. L.; Koenigs, P.; Porter, N.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 8042.

(6) (a) Noren, C. J.; et al. *Science* **1989**, *244*, 182. (b) Ellman, J. A.; et al. *Methods Enzymol.*, in press.

(7) Recently a similar approach has been used to incorporate unnatural amino acids into peptides; see: Bain, J. D.; et al. *J. Am. Chem. Soc.* **1989**, *111*, 8013.

(8) (a) Tsugita, A.; et al. *J. Biol. Chem.* **1968**, *243*, 391. (b) Tsugita, A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 3, pp 343-411 and references therein.

(9) Weaver, L. H.; Matthews, B. W. *J. Mol. Biol.* **1987**, *193*, 189.

(10) Dao-Pin, S.; Liao, D.-I.; Remington, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5361 and references therein.

(11) Pillai, V. N. R. *Synthesis* **1990**, 1.

(12) The cysteine-free mutant T4L (C54T, C97A) was used in these studies because it is virtually identical with the wild-type protein in structure and activity, but does not suffer oxidative degradation; see: Perry, L. J.; Wetzel, R. *Biochemistry* **1986**, *25*, 733.

(13) (a) Zubay, G. *Annu. Rev. Genet.* **1973**, *7*, 267. (b) Collins, J. *Gene* **1979**, *6*, 29. (c) Pratt, J. In *Transcription and Translation: A Practical Approach*; Hames, B. D., Higgins, S. J., Eds.; IRL Press: Oxford, 1984; pp 179-209.

0002-7863/91/1513-2758$02.50/0 © 1991 American Chemical Society

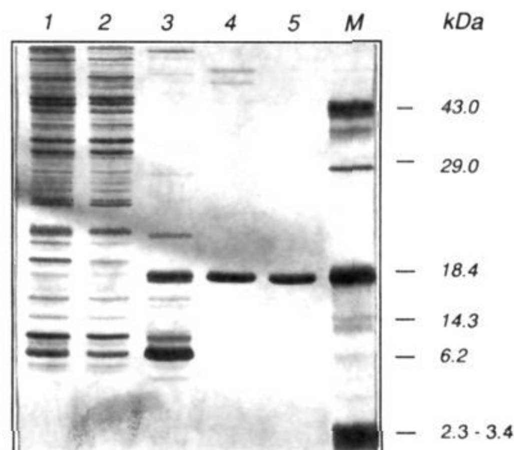


Figure 1. Course of T4 lysozyme purification (adapted from ref 12) from cell-free *E. coli* extract analyzed by 15% SDS-PAGE. Lanes: 1, crude polyethylenimine lysate supernatant; 2, DEAE- and CM-cellulose tandem column wash; 3, pooled T4L-active fractions from NaCl gradient on CM-cellulose column; 4, purified in vitro produced T4L after cation-exchange chromatography (Pharmacia Mono S); 5, T4L purified from IPTG-induced *E. coli* cells harboring the plasmid pHSe54,97.TA; 6, molecular weight standards. In vitro protein synthesis reactions were as described in refs 6 and 13.

plasmid pHSe54,97.TA which encodes T4L behind the twin *tac* promoter (Figure 1). Both in vitro and in vivo preparations of T4L have the same chromatographic properties and apparent molecular mass of approximately 18 700 Da (daltons). More importantly, both preparations display the same kinetics toward lysis of *E. coli* strain Nap IV[14] cells at three different substrate concentrations (data not shown).

The amber mutant [D20 → TAG (pT4LD20am)] of T4L was constructed via the Eckstein method.[15] When the in vitro protein synthesis system is programmed with plasmid pT4LD20am in the absence of suppressor tRNA, no synthesis of T4L is seen as determined by polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled proteins (lane 2, Figure 3). Based on scintillation counting of gel slices, less than 1.5% readthrough of the amber codon is observed at 2 mM added Mg^{2+}. This control shows that the level of translation of the amber codon by noncognate tRNAs is insignificant under the conditions employed. Addition of 7.5 μg of full-length unacylated suppressor $tRNA_{CUA}$ (Figure 2) to the pT4LD20am-programmed reaction again results in less than 1.5% readthrough at 2 mM added Mg^{2+} (Figure 3, lane 3). This control insures that protein produced from the amber mutant will not be heterogeneous due to partial incorporation of the natural 20 amino acids. In accord with the lack of a full-length T4L band, neither of these two control reactions yielded any lytic activity. In contrast, in vitro synthesis reactions primed with plasmid pHSe54,97.TA produced 40–50 μg of enzyme/mL of in vitro reaction mixture (lane 1, Figure 3). The wild-type in vitro reaction hydrolyzes *E. coli* cells at rates of approximately 4 OD_{450nm} units/min per 2 μL of in vitro supernatant.[14]

(14) Nelson, M. A.; Gold, L. *Mol. Gen. Genet.* **1982**, *188*, 69. Supernatants (2 μL) from suppression reactions were mixed with 1.0 mL of a ~500 μg mL^{-1} suspension of *E. coli* cells in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, and the change in absorbance at 450 nm was plotted as a function of time.

(15) Sayers, J. R.; Schmidt, W.; Eckstein, F. *Nucleic Acids Res.* **1988**, *16*, 791. The mutant pT4LD20am was constructed by first inserting the ~650-bp *Bam*HI–*Hin*dIII fragment of pHSe54,97.TA into RF M13mp18 and mutating the coding strand with oligonucleotide 5' GCCTTCTGTCTATTTATA-GATTTT 3' (mismatches underlined). After screening, the mutant-containing duplex *Bam*HI–*Hin*dIII was recloned into the pHSe expression vector to give pT4LD20am, and the entire T4L gene was sequenced.

(16) Bruce, A. G.; Uhlenbeck, O. C. *Biochemistry* **1982**, *21*, 855.

(17) Anthony-Cahill, S. J. Ph.D. Thesis, University of California, Berkeley, 1990.

(18) Feinberg, R. S.; Merrifield, R. B. *Tetrahedron* **1972**, *28*, 5865.

(19) Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.*, in press.

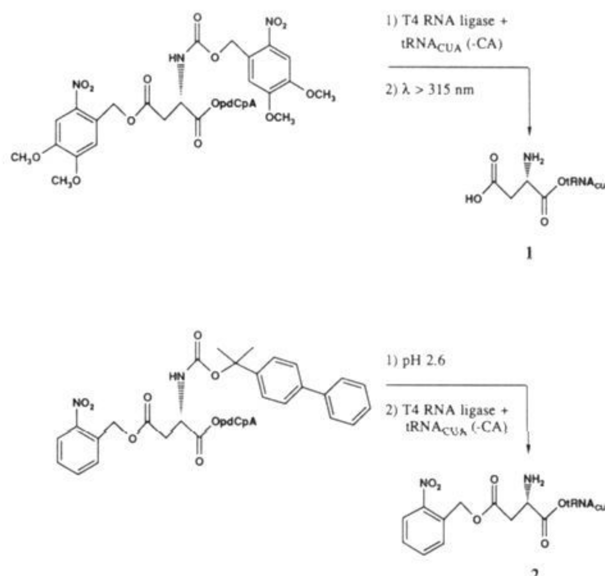


Figure 2. Aminoacyl suppressor $tRNA_{CUA}$ construction scheme. Yeast $tRNA^{Phe}$ containing CUA at the anticodon loop and missing the 3' terminal CA residues ($tRNA_{CUA}$ (–CA)) was constructed according to Bruce and Uhlenbeck[16] using synthetic rCUAA.[17] NVOC-protected (nitrovertryloxycarbonyl) or BPOC-protected[18] (biphenylisopropyloxy-carbonyl) aminoacyl pdCpA was synthesized and purified according to ref 19 and was then ligated to $tRNA_{CUA}$ (–CA). Asp-$tRNA_{CUA}$ (**1**) and NB-Asp-$tRNA_{CUA}$ (**2**) were added to the suppression reactions of Figure 3. Full-length unacylated $tRNA_{CUA}$ was prepared by ligating pdCpA to $tRNA_{CUA}$ (–CA).

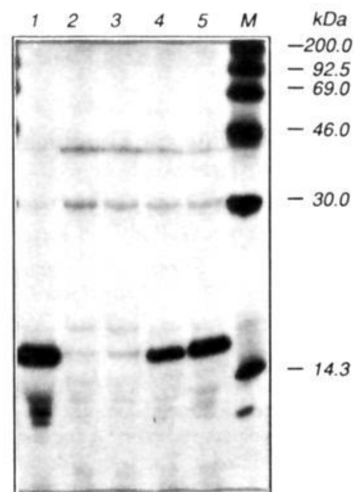


Figure 3. Autoradiogram of in vitro suppression reactions labeled with [^{35}S]methionine and containing the following plasmids and tRNAs: lane 1, pHSe54,97.TA (wild type); 2, pT4LD20am without $tRNA_{CUA}$; 3, pT4LD20am and 7.5 μg of full-length unacylated $tRNA_{CUA}$; 4, pT4LD20am and 7.5 μg of **1**; 5, pT4LD20am and 7.5 μg of **2**. Lane M contains ^{14}C-methylated molecular weight standards. Intense bands of 18.7 kDa mass correspond to T4L, and faint bands of 30 kDa mass correspond to β-lactamase (pHSe54,97.TA also contains the β-lactamase marker). Cleared supernatants (10 μL) from terminated 30-μL in vitro reactions were incubated with 2 μL of 2.5 mg mL^{-1} RNase A from 15 min at 37 °C and then analyzed by 15% SDS–PAGE. Each 30-μL reaction contained 7 μCi of [^{35}S]methionine.

When supplemented with 7.5 μg of Asp-$tRNA_{CUA}$, the pT4LD20am-primed system yielded a significant amount of material corresponding to T4L (Figure 3, lane 4). Furthermore, material produced in this reaction hydrolyzed *E. coli* cells at about 27 ± 3% of the rate of the wild-type reaction. Scintillation counting of the bands from lanes 1 and 4 (Figure 3) gave a suppression efficiency of 22 ± 2%. Close agreement between recovered activity and amount of material produced argues

strongly that Asp-tRNA$_{CUA}$ accurately delivers aspartic acid to amber mutant 20 of T4L.

Suppression experiments were then carried out with the caged aspartyl β-nitrobenzyl ester. When the pT4LD20am-programmed in vitro protein synthesis reaction was supplemented with 7.5 μg of NB-Asp-tRNA$_{CUA}$, a T4L of the correct molecular weight was produced (lane 5, Figure 3). The NB-Asp-suppressed pT4LD20am produced about 37 ± 4% of full-length T4L compared to wild-type control, and the caged protein was catalytically inactive. However, the 2-nitrobenzyl group can be easily removed by photolysis: irradiation of the crude in vitro reaction supernatants (lane 5, Figure 3) at $\lambda > 315$ nm (Oriel Hg–Xe arc lamp, 1000 W) restored lytic activity to the NB-Asp-suppressed T4L. The irradiated material yielded 32 ± 3% of the activity of the wild-type enzyme, agreeing very closely with suppression levels based on scintillation counting of gel bands. These results show that NB-Asp-tRNA$_{CUA}$ efficiently suppresses pT4LD20am to produce mutant T4L with latent activity that can be released by rapid photolysis. Moreover, virtually all of the caged protein is converted to the active form. We are currently extending this methodology to other amino acids such as serine, tyrosine, lysine, and cysteine located at both internal and external sites in a number of proteins.

Acknowledgment. We thank the Matthews and Dahlquist groups and Laurence McIntosh of the University of Oregon for providing the pHSe expression vectors and maps and Nora Linderoth for the Nap IV strain. This work was funded by the Procter and Gamble UERP program, ONR Grant N00014-87-K-0256 (J.A.E.), and by the Director, Office of Energy Research, Office of Basic Energy Sciences, Division of Material Sciences and also by the Division of Energy Biosciences of the U.S. Department of Energy (DE-AC03-76SF00098) (D.M.). D.M. was supported by American Cancer Society postdoctoral fellowship PF-4014 and J.A.E. by National Science Foundation postdoctoral fellowship CHE-8907488. P.G.S. is a W. M. Keck Foundation Investigator.

Second-Sphere Coordination of Transition-Metal Complexes by Calix[4]arenes

Jerry L. Atwood,* G. William Orr, Fumio Hamada, Rebecca L. Vincent, Simon G. Bott, and Kerry D. Robinson

Department of Chemistry, University of Alabama
Tuscaloosa, Alabama 35487
Received December 17, 1990

Second-sphere coordination has been defined by Stoddart as the "non-covalent bonding of chemical entities to the first coordination sphere of a transition metal complex."[1b] The importance of such systems for biological receptors[2–4] and for supramolecular assemblies[5] has been noted.[1] The use of crown ethers as second-sphere ligands via hydrogen bonding is well documented.[6,7] Numerous studies of cyclodextrins coordinated to transition-metal complexes have also appeared.[1,7,8–14] However, even though

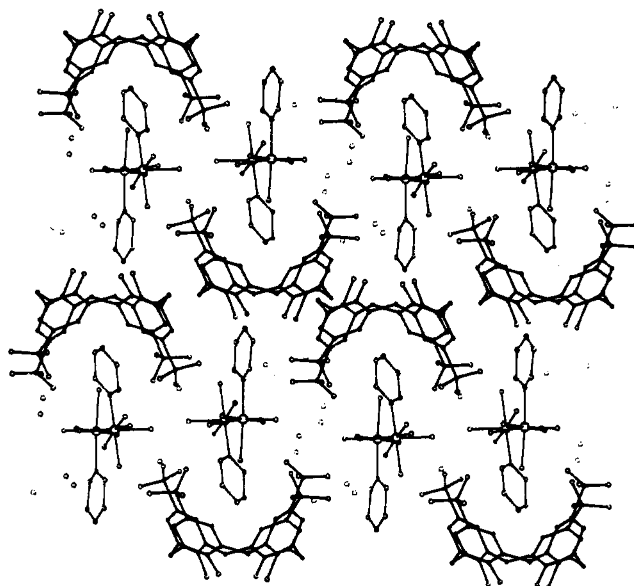


Figure 1. Structure of $[(H_2O)_5Ni(NC_5H_5)]_2(Na)$[calix[4]arene sulfonate]$\cdot 3.5H_2O$ illustrating the bilayer arrangement of the calixarenes, the intercalation into the bilayer, and the second-sphere coordination of the transition-metal complex. The two independent Ni–N distances are 2.08 (1) Å, and the Ni–O(water) lengths range from 2.05 (1) to 2.18 (1) Å.

calixarenes are one of the most readily accessible three-dimensional macrocycles[15] and are under intense investigation by numerous groups,[16–18] no examples of second-sphere coordination have appeared. We report herein that the hydrophobic cavity of [calix[4]arene sulfonate]$^{5-}$ can be employed for such second-sphere coordination. In addition, the title compounds also exhibit a new type of intercalation behavior with regard to the bilayer assembly of the calixarenes.[19–21]

Three equivalents of the deep blue $Ni(NC_5H_5)_3(NO_3)_2$[22,23] was dissolved in deionized water (with hydrolysis) to which 1 equiv of Na_5[calix[4]arene sulfonate][19] had been added. The resultant pale blue solution yielded pale blue crystals of $[(H_2O)_5Ni(NC_5H_5)]_2(Na)$[calix[4]arene sulfonate]$\cdot 3.5H_2O$ upon slow evaporation in a vacuum desiccator. The structure[24] of the complex is shown in Figure 1.

There are two different $[(H_2O)_5Ni(NC_5H_5)]^{2+}$ cations in the assembly. In one the hydrophobic pyridine ring is complexed by the hydrophobic cavity of the calixarene. The depth of penetration of the aromatic moiety into the cavity may be measured by the distance of the centroid of the pyridine ring from the plane of the calixarene CH_2 carbon atoms. For the nickel complex the value

(1) (a) Colquhoun, H. M.; Stoddart, J. F.; Williams, D. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1986. (b) Alston, D. R.; Ashton, P. R.; Lilley, T. H.; Stoddart, J. F.; Zarzycki, R.; Slawin, A. M. Z.; Williams, D. J. *Carbohydr. Res.* **1989**, *192*, 259.
(2) Shaw, J.; Everett, G. W. *Inorg. Chem.* **1985**, *24*, 1917.
(3) Gessner, R. V.; Quigley, G. J.; Wang, A. H.-J.; van der Marel, G. A.; van Boom, J. H.; Rich, A. *Biochemistry* **1985**, *24*, 237.
(4) Sherman, S. E.; Lippard, S. J. *Chem. Rev.* **1987**, *87*, 1153.
(5) Lehn, J.-M. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 89.
(6) Colquhoun, H. M.; Doughty, S. M.; Slawin, A. M. Z.; Stoddart, J. F.; Williams, D. J. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 135.
(7) Alston, D. R.; Slawin, A. M. Z.; Stoddart, J. F.; Williams, D. J. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 786.
(8) Joh, T.; Harada, A.; Takahashi, S. *Mem. Inst. Sci. Ind. Res., Osaka Univ.* **1989**, *46*, 37.
(9) Alston, D. R.; Slawin, A. M. Z.; Stoddart, J. F.; Williams, D. J.; Zarzycki, R. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1184.
(10) Alston, D. R.; Lilley, T. H.; Stoddart, J. F. *J. Chem. Soc., Chem. Commun.* **1985**, 1600.
(11) Kobayashi, N.; Opallo, M. *J. Chem. Soc., Chem. Commun.* **1990**, 477.
(12) Klingert, B.; Rihs, G. *Organometallics* **1990**, *9*, 1135.
(13) Harada, A.; Hu, Y.; Yamamoto, S.; Takahashi, S. *J. Chem. Soc., Dalton Trans.* **1988**, 729.
(14) Ashton, P. R.; Stoddart, J. F.; Zarzycki, R. *Tetrahedron Lett.* **1988**, *29*, 2103.
(15) Gutsche, C. D. *Calixarenes*; Royal Soc. Chem.: Cambridge, 1989.
(16) *Calixarenes*; Böhmer, V., Vicens, J., Eds.; Kluwer: Dordrecht, 1990.
(17) Gutsche, C. D. In *Inclusion Compounds*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Eds.; Oxford University Press: Oxford; Vol. 4, 1991; pp 27–63.
(18) Andreetti, G. D.; Ugozzoli, F.; Ungaro, R.; Pochini, A. In *Inclusion Compounds*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Eds.; Oxford University Press: Oxford; Vol. 4, 1991; pp 64–127.
(19) Coleman, A. W.; Bott, S. G.; Morley, S. D.; Means, C. M.; Robinson, K. D.; Zhang, H.; Atwood, J. L. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1361.
(20) Bott, S. G.; Coleman, A. W.; Atwood, J. L. *J. Am. Chem. Soc.* **1988**, *110*, 610.
(21) Atwood, J. L.; Coleman, A. W.; Zhang, H.; Bott, S. G. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1989**, *7*, 203.
(22) Rosenthal, M. R.; Drago, R. S. *Inorg. Chem.* **1965**, *4*, 840.
(23) Atwood, J. L.; Bott, S. G.; Vincent, R. L. *J. Crystallogr. Spectrosc. Res.* **1990**, *20*, 631.
(24) $[(H_2O)_5Ni(NC_5H_5)]_2(Na)$[calix[4]arene sulfonate]$\cdot 3.5H_2O$ crystallizes in the triclinic space group $P\bar{1}$ with $a = 12.487$ (4) Å, $b = 14.281$ (2) Å, $c = 15.055$ (5) Å, $\alpha = 85.66$ (2)°, $\beta = 80.07$ (2)°, $\gamma = 80.48$ (2)°, and $D_{calcd} = 1.64$ g cm^{-3} for $Z = 2$. Refinement based on 2441 observed reflections led to a final R value of 0.066.

0002-7863/91/1513-2760$02.50/0 © 1991 American Chemical Society