Concluding Remarks. New synthetic strategies have been developed for the dichromium(II) and ditungsten(II) complexes with ligands containing the very basic bridging group -NCHN-. Extensive application to the tungsten system may be expected since this is the first successful isolation of such a ditungsten(II) complex. The metal-metal quadruple bond is well established on the basis of the molecular structure and consistent spectroscopic data. The accessibility of the cation $W_2(DFM)_4^+$ is clearly suggested by the electrochemical study, and attempts to isolate it will be undertaken.

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Supplementary Material Available: Complete tables of crystal data, positional parameters, bond distances and angles, and anisotropic displacement parameters for structures **1** and **2** (19 pages); listing of observed and calculated structure factors (19 pages). Ordering information is given on any current masthead page.

Antibody Targeting to Bacterial Cells Using Receptor-Specific Ligands

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Abstract: The binding of antibodies to bacterial cells is necessary to effect host defense mechanisms such as complement activation and macrophage recognition. We have designed a biotinylated *C*-glycoside of mannose (BCM) that, when complexed with avidin, targets antibodies to a pathogenic strain of *Escherichia coli* containing type 1 pili mannose-specific receptors. Antibody binding to the bacterial cells was viewed by transmission electron microscopy using a protein A gold label. Antibody binding occurs only in the presence of the BCM-avidin conjugate and can be titrated off the surface of the cell by methyl α-D-mannopyranoside, demonstrating that antibody binding is mediated by the receptor. The conserved binding domain of cell-surface lectins can therefore be utilized to direct antibodies to pathogens.

Introduction

The binding of antibodies to antigenic determinants on the surface of a bacterial pathogen is required to activate pathways in humoral and cellular immunity.[1-4] The process of antibody-mediated cell toxicity includes complement activation (classical pathway) and opsonization for macrophage-mediated endocytosis along with direct recognition of the antibody-coated bacterium by macrophage Fc receptors.[5,6] Several strains of enterobacteria, such as *Escherichia*, *Klebsiella*, *Shigella*, and *Salmonella*, have proteinaceous appendages called pili (or fimbriae) that are located on the surface of the organism and provide antigenic recognition sites for the host's immune system.[4,7] Type 1 pili also contain receptors specific for terminal α-linked mannosides which mediate the adhesion of bacteria to host cells, a process that is essential for infectivity.[8-14] Many bacterial lectins, however, undergo rapid genetic changes to evade the host's immune system. Despite these genetic changes, the biological function and thus the binding specificity of bacterial cell-surface lectins are conserved.[4] We describe herein a strategy to target antibodies to a pathogenic strain of *Escherichia coli* K1 by exploiting the ligand specificity of the conserved type 1 pili mannose-specific receptors.

Both complement factor C1q and macrophage cells recognize an antibody-coated particle by interacting with the Fc region of the antibodies. Capon et al. and Traunecker et al. have shown that genetically engineered proteins in which the binding determinants of IgG or IgM molecules are replaced with CD4 can be used to target the Fc region to HIV-infected cells.[15,16] Shokat and Schultz have recently demonstrated that antibodies can be directed to purified HIV glycoprotein gp120 via 2,4-dinitrophenol conjugates of CD4 in vitro.[17] We report here that (1) the conjugate of a biotinylated *C*-glycoside of mannose (BCM; Figure 1) with avidin can mediate the attachment of anti-avidin antibodies to bacterial cells through the type 1 pili mannose-specific receptors and (2) the antibody binding event can be directly assayed using transmission electron microscopy (TEM) on whole bacterial cells. Since this approach to antibody targeting does not involve molecular cloning, it can be applied to other pathogenic organisms for which the cell-surface receptors bind to small molecules. Furthermore, the antibody binding event is visualized directly on the surface of the cell rather than on a purified receptor. Direct observation of antibody-coated pathogens by TEM should provide

(1) Joiner, K. A.; Brown, E. J.; Frank, M. M. *Annu. Rev. Immunol.* **1984**, *2*, 461.
(2) Soderstrom, T.; Ohman, L. *Scand. J. Immunol.* **1984**, *20*, 299.
(3) Ofek, I.; Sharon, N. *Infect. Immun.* **1988**, *56*, 539.
(4) Virji, M.; Heckels, J. E. *Infect. Immun.* **1985**, *49*, 621.
(5) Reid, K. B. M.; Porter, R. R. *Annu. Rev. Biochem.* **1981**, *50*, 433.
(6) Silverstein, S. C.; Steinman, R. M.; Cohn, Z. A. *Annu. Rev. Biochem.* **1977**, *46*, 669. Duncan, A. R.; Woof, J. M.; Partridge, L. J.; Burton, D. R.; Winter, G. *Nature* **1988**, *332*, 563.
(7) Clegg, S.; Gerlach, G. F. *J. Bacteriol.* **1987**, *169*, 934.
(8) Firon, N.; Ofek, I.; Sharon, N. *Infect. Immun.* **1984**, *43*, 1088.
(9) Firon, N.; Ofek, I.; Sharon, N. *Carbohydr. Res.* **1983**, *120*, 235.
(10) Ofek, I.; Mirelman, D.; Sharon, N. *Nature* **1977**, *265*, 623.
(11) Eshdat, Y.; Ofek, I.; Yashouv-Gan, Y.; Sharon, N.; Mirelman, D. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1551.
(12) Hanson, M. S.; Brinton, C. C., Jr. *Nature* **1988**, *332*, 265.
(13) Hanson, M. S.; Hempel, J.; Brinton, C. C., Jr. *J. Bacteriol.* **1988**, *170*, 3350.
(14) Bloch, C. A.; Orndorff, P. E. *Infect. Immun.* **1990**, *58*, 275.
(15) Capon, D. J.; et al. *Nature* **1989**, *337*, 525. Byrn, R. A.; et al. *Nature* **1990**, *344*, 667.
(16) Traunecker, A.; Schneider, J.; Kiefer, H.; Karjalainen, K. *Nature* **1989**, *339*, 68.
(17) Schultz, P. G.; Shokat, K. *J. Am. Chem. Soc.* **1991**, *113*, 1861.

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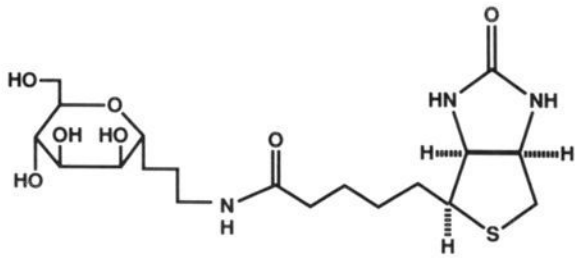

Figure 1. Biotinylated *C*-glycoside of mannose (BCM).

information on the nature of the cell-surface interactions that are necessary to achieve cell killing.

Results and Discussion

In order to target antibodies to the *E. Coli* cells, we required a high-affinity ligand that recognizes the type 1 pili mannose receptor and contains an antibody binding domain. To achieve this goal, we have synthesized a biotinylated *C*-glycoside of mannose, BCM (Figure 1), that binds to bacterial mannose-specific receptors.[18] This compound was designed to mediate the attachment of streptavidin or avidin to the surface of bacterial cells. BCM was also designed with a carbon atom rather than an oxygen atom at the glycosidic position to provide resistance to chemical and enzymatic degradation while maintaining receptor binding activity.[18] While the binding affinity of the mannose receptor to a single mannosyl ligand is fairly weak (K_D is in the low millimolar range[8,11]), we felt that a multivalent ligand would be capable of targeting antibodies to the organism at concentrations in the micromolar range. The tetrameric structures of streptavidin and avidin provide such multivalency when BCM is bound at the four biotin-binding sites.

The conjugates of BCM with streptavidin and avidin were formed by incubating a 10-fold excess of BCM with each protein overnight at 0 °C. The solutions were then dialyzed against PBS (3 × 400 mL, 6000–8000 MW cutoff), and the total protein concentrations were determined by BCA protein assay (Pierce) and $E^{1\%}_{280}$. BCM and its conjugates with streptavidin and avidin were assayed for bacterial receptor binding using agglutination studies with yeast cells.[19] The bacterial strain used in our study was a systemically invasive *E. coli* K1 *pilA+::tetR* that is responsible for sepsis and meningitis in human infants.[14] Total inhibition of agglutination by BCM was achieved at a concentration of 7 mM as compared to approximately 0.6 mM for the complex of BCM with streptavidin.[20] Although the tetrameric BCM–streptavidin conjugate binds to the bacterial receptors with higher avidity than BCM alone, we felt that the enhanced avidity was not sufficient to allow antibody targeting at micromolar concentrations. Furthermore, the limited solubility of streptavidin under the conditions of these assays created problems in subsequent antibody targeting experiments.

Egg white avidin, a more soluble protein, contains mannosyl moieties as part of its oligosaccharide component.[21] Due to its glycosylation pattern, avidin alone binds to the bacterial receptors (total inhibition of agglutination by avidin is achieved at a concentration of 0.4 mM). However, the BCM–avidin conjugate inhibits agglutination at a concentration that is 1 order of magnitude lower than avidin alone (total inhibition occurs at 0.05 mM for the complex of BCM with avidin[18]). The increased avidity of the BCM–avidin complex for the bacterial receptors suggests that BCM is binding both avidin and the mannose receptors on the pili. Since avidin contains four BCM binding sites as well as mannosylated oligosaccharides, the resulting conjugate binds more tightly to the bacterial receptors than a monomeric ligand and serves as a more effective antibody targeting agent than the BCM–streptavidin complex.

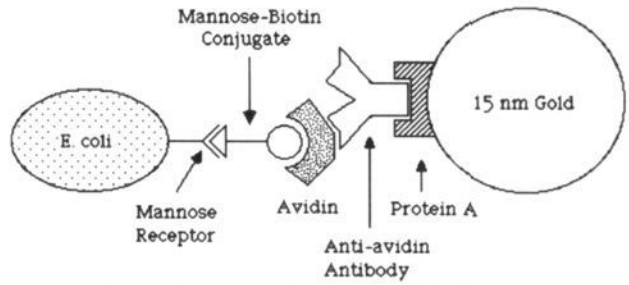

Figure 2. Schematic representation of the antibody binding assay. The BCM–avidin complex binds to mannose receptors on the bacterial pili. Anti-avidin antibodies bind to avidin, and protein A conjugated to 15-nm gold particles binds to the Fc region of the antibody. The colloidal gold particle is visualized by transmission electron microscopy. The BCM–avidin conjugate is depicted as monomeric for illustrative purposes.

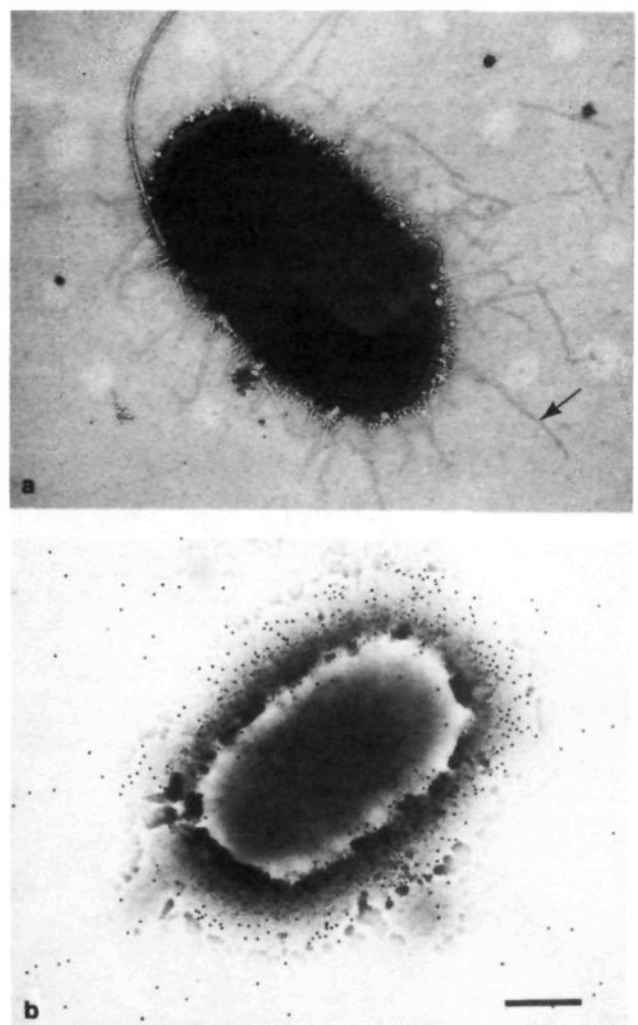

Figure 3. Transmission electron micrographs of bacterial cells with and without colloidal gold labeling. The protocol used to generate samples for microscopy is described in the Experimental Section. (a) Negatively stained bacterial cell. The arrow shows the pili protruding from the surface. (b) Bacterial cell treated with the BCM–avidin–antibody complex (10-min incubation) followed by protein A–15 nm colloidal gold conjugate (GpA) (10 min). Note the density of gold particles bound in the region of the pili. Bar, 0.5 μm.

(18) The synthesis of BCM is described in detail in: Bertozzi, C. R.; Bednarski, M. D. *Carbohydr. Res.*, in press.

(19) For references describing the use of bacterial agglutination assays with yeast cells, see ref 9 and: Firon, N.; Ofek, I.; Sharon, N. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 1426. The concentrations necessary to obtain total inhibition of agglutination were measured as described in the Experimental Section. Although these concentrations do not represent binding constants, they can be used to assess qualitatively the relative avidities of the test compounds for the bacterial receptors.

(20) The BCM–streptavidin conjugate was partially insoluble at this concentration.

(21) Huang, T.-S.; DeLange, R. J. *J. Biol. Chem.* **1971**, *246*, 686.

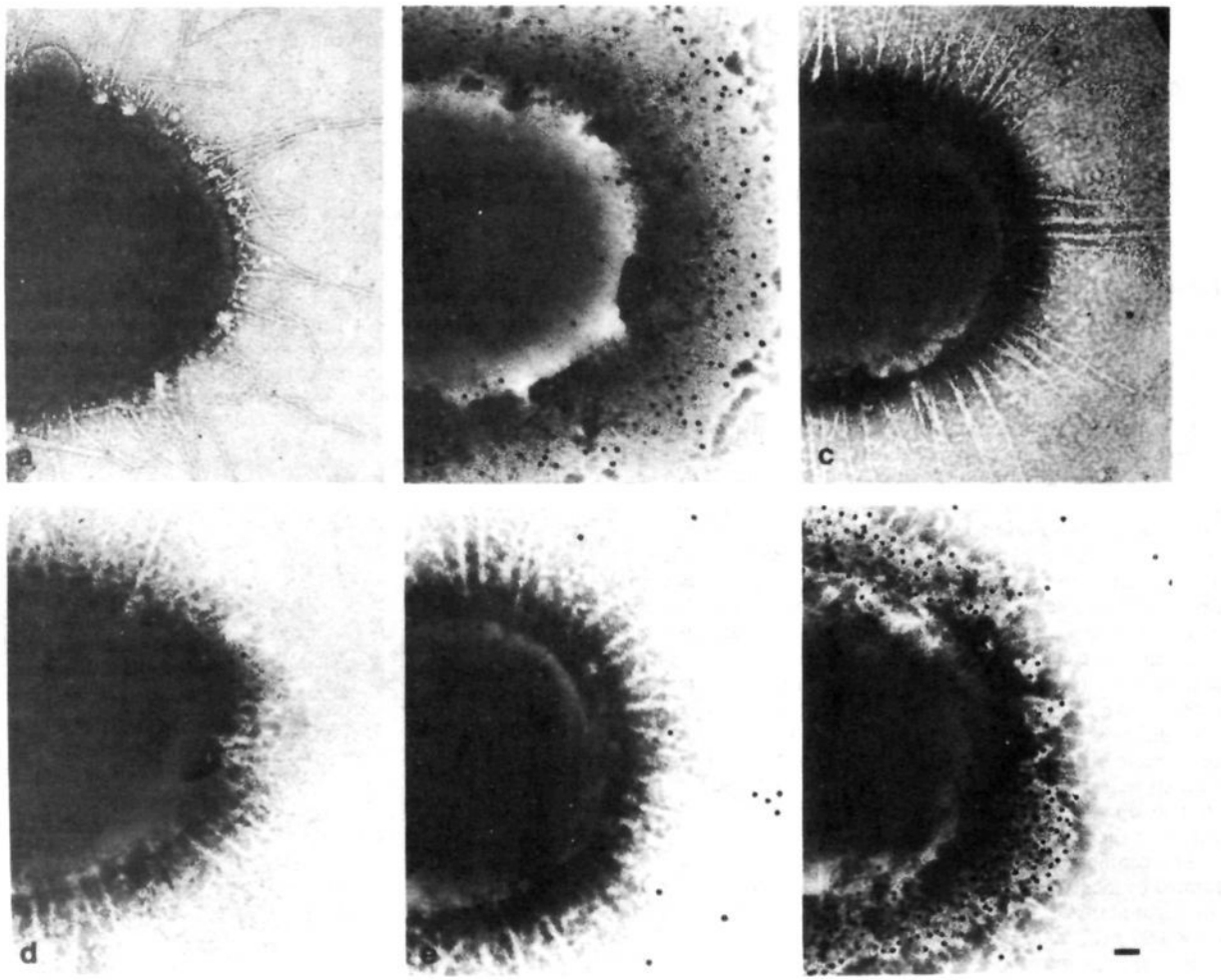

Figure 4. Transmission electron micrographs of bacterial cells showing the cell-surface region. (a) An enlargement of the cell shown in Figure 3a emphasizing the tip of the cell for comparison with control samples. (b) A similar enlargement of the cell shown in Figure 3b. (c) Control sample in which the BCM–avidin conjugate was omitted. Only a background level of gold binding is seen. (d) Control sample in which anti-avidin IgG has been omitted. (e) Control sample in which the conditions used in (b) were repeated in the presence of 100 mM methyl α-D-mannopyranoside. The antibodies do not bind to the pili since the receptors are blocked with mannose. (f) Control sample in which the conditions used in (b) were repeated in the presence of 100 mM methyl α-D-glucopyranoside. Since glucose does not bind to the receptors, antibody binding is unaffected. Bar, 0.1 μm.

The conjugate of BCM with avidin was then utilized to target anti-avidin antibodies to the bacterial cell surface. Localization of anti-avidin antibodies on the bacterial cell was assayed by transmission electron microscopy using a protein A colloidal gold label as shown schematically in Figure 2.[22,23] The *E. coli* K1 cells were deposited onto Formvar-coated, carbon-stabilized copper grids and incubated with the BCM–avidin conjugate complexed with monoclonal anti-avidin antibodies.[24] The grids were then treated with a solution of protein A–15 nm gold conjugate (GpA) in PBS containing 1% BSA,[25] washed three times with PBS, and negatively stained with 1% phosphotungstic acid (pH 6.9). Figure 3a shows an untreated bacterial cell. The pili are seen as long fibers protruding from the organism (indicated with an arrow), and each pilus contains several mannose-specific receptors. Figure 3b shows a similar cell treated with the BCM–avidin–antibody complex followed by GpA as described above. A high density of gold particles can be seen bound at the surface of the organism in the region of the pili. The number of gold particles bound to the surface of the cell is related to the density of antibodies attached to the pathogen.[26] Parts a and b of Figure 4 show enlargements of the regions at the outer edge of the cells in Figure 3a,b. Control samples lacking either the BCM–avidin complex (Figure 4c) or anti-avidin antibodies (Figure 4d) show no pattern of gold binding to the pathogen, demonstrating that only in the presence of the BCM–avidin ligand do antibodies recognize the bacteria. Furthermore, when avidin without bound BCM is used in the presence of anti-avidin antibodies, a large reduction in bound gold particles is seen, suggesting that although avidin binds to the mannose receptors, the avidity of avidin alone is not sufficient for strong antibody targeting. Antibody binding to the bacterial pili can also be inhibited by blocking the receptors with methyl α-D-mannopyranoside (100 mM) (Figure 4e). Methyl α-D-gluco-

(22) The BCM–avidin complex is depicted as monomeric for illustrative purposes. The actual complex makes several contacts with mannose receptors on the bacterium and with antibody molecules. The result is the formation of BCM–avidin–antibody clusters that bind with high avidity to the bacterial cell.

(23) For reviews on immunogold staining of bacterial pili, see the following: (a) Smit, J.; Todd, W. J. In *Ultrastructure Techniques for Microorganisms*; Aldrich, H. C., Todd, W. J., Eds.; Plenum: New York, 1986; pp 469–516. (b) Robinson, E. N., Jr.; et al. *Infect. Immun.* **1984**, *46*, 361.

(24) The BCM–avidin–antibody complex was formed prior to use in the assay. A significant decrease in the density of gold particles bound to the pathogen was observed when the grids were incubated with the BCM–avidin complex followed by anti-avidin antibodies in a separate step. Several levels of multivalency (the bivalency of the antibody as well as the tetravalency of avidin) increase the avidity of the resulting antibody-bound clusters to the pathogen such that subsequent washing steps do not result in a significant decrease in bound BCM–avidin–antibody complex.

(25) BSA acts as an in situ blocking reagent to minimize nonspecific adhesion of the colloidal gold to the TEM grid.

(26) Negative staining in transmission electron microscopy involves the deposition of a thin film of heavy-atom stain over the contours of the object. Since the pili have a width of only 70 Å, when the pili are surrounded with protein or gold clusters of similar size, they are no longer defined by negative staining and may be difficult to discern.

pyranoside, which does not bind to the receptor, had no effect on antibody binding as measured by gold localization at the cell surface (Figure 4f). These results demonstrate that antibodies can be targeted to the *E. coli* specifically through their mannose receptors.

In summary, we have exploited a carbohydrate receptor to target antibodies to the surface of a pathogenic strain of *E. coli* that would not otherwise recognize the organism. Carbohydrate–protein conjugates similar to BCM–avidin may be used as a general strategy to prime pathogenic organisms for killing by host defense mechanisms. The use of these conjugates to mediate cell killing by complement proteins and macrophage cells is currently being investigated.

Experimental Section

Agglutination Assays. *E. coli* K1 *pilA+::tetR* was grown for 24 h at 37 °C on solid LB media supplemented with tetracycline and suspended with a cotton swab in 4 mL of Dulbecco's PBS to a final dilution of 2×10^8 cells/mL. Yeast (*Saccharomyces cerevisiae*, wild type) was grown for 36 h on solid YPD media at 30 °C and suspended with a cotton swab in 4 mL of Dulbecco's PBS to a final dilution of 1×10^8 cells/mL. Protein concentrations were determined by BCA Protein Assay (Pierce). Agglutination assays were performed on a 20-well ceramic ring plate. Typically, 90 μL of a solution of the test compound was combined with 30 μL of the bacterial suspension. After 30 s, 30 μL of the yeast suspension was added to give a final volume of 150 μL and the wells were allowed to develop for 3 min with agitation. A 5-μL aliquot was removed from each well and spread onto a standard microscope slide. The slides were quickly heat fixed and mounted with 10 μL of glycerol. The slides were examined under phase contrast at 500× magnification using a Zeiss Axioskop microscope. Agglutination was observed as clusters of cells. Total inhibition of agglutination was determined by the observation of single cells only. Determination of the concentrations necessary for total inhibition of agglutination was accurate within 20%.

Transmission Electron Microscopy. *E. coli* K1 *pilA+::tetR* was cultivated on solid LB media containing tetracycline at 37 °C for 18–20 h and suspended in PBS using a sterile swab to a final dilution of 10^9 cells/mL immediately prior to use. TEM samples were prepared in the following manner. Copper electron microscopy specimen grids (200 mesh) were coated with polyvinyl Formvar, carbon stabilized, and ionized by argon plasma discharge. A drop of the bacterial suspension was placed onto the grids, and after 2 min the drop was removed via pipet and the excess fluid blotted with filter paper. The grids were washed three times with PBS before negative staining with a 1% solution of phosphotungstic acid (pH 6.9). All specimens were examined with a Zeiss EM-109 electron microscope. The BCM–avidin conjugate was used as a 1.5×10^{-6} M solution in PBS. Monoclonal anti-avidin IgG (Sigma) (1.2×10^{-5} M in PBS) was preincubated with the BCM–avidin conjugate overnight at 0 °C before use. Protein A–15 nm colloidal gold conjugate (GpA, E-Y Laboratories) was diluted 1:10 in PBS containing 1% BSA.

Acknowledgment. We thank Dr. C. A. Bloch (Stanford University Medical Center) and Professor P. E. Orndorff (North Carolina State University) for their gift of *E. coli* K1 *pilA+::tetR* and Merck Sharpe & Dohme for their generous gift of streptavidin. We also thank Kevan Shokat and Peter Schultz (University of California, Berkeley) for many helpful discussions and Caroline Schooley at the Robert D. Ogg Electron Microscope Laboratory (University of California, Berkeley) for her technical advice. This research was supported by National Institute of Health Award No. R29 GM43037-02 and the Procter & Gamble University Exploratory Research Program. C.R.B. thanks the Office of Naval Research (ONR) and AT&T Bell Laboratories for graduate fellowships. M.D.B. thanks the American Cancer Society for a Junior Faculty Award.

α- and β-Deuterium Kinetic Isotope Effects on the Inactivation of the General Acyl-Coenzyme A Dehydrogenase from Pig Kidney by (2-Methylenecyclopropane)acetyl-CoA

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Abstract: Seven deuterium-labeled and unlabeled forms of (2-methylenecyclopropane)acetyl-CoA (MCPA-CoA) have been employed in kinetic studies to assess α- and β-deuterium isotope effects on the inactivation of the general acyl-CoA dehydrogenase from pig kidney. The racemic forms of α-d_2-MCPA-CoA and β-*d*-MCPA-CoA do not show any significant kinetic isotope effects. When inactivations of the enzyme caused by (*R*)-MCPA-CoA and by (*R*)-β-*d*-MCPA-CoA or those involving (*S*)-MCPA-CoA and (*S*)-β-*d*-MCPA-CoA are compared, only slight secondary deuterium isotope effects on the time versus extent of inactivation profiles are detected. These observations mitigate against one proposed mechanism for the inactivation which postulates dehydrogenation of the thioester substrate to form (2-methylenecyclopropylidene)acetyl-CoA, a reactive electrophilic Michael acceptor.

Introduction

Over the past decade there has been a growing interest in the chemistry of acyl-CoA dehydrogenases and in the biochemistry and molecular biology associated with various acyl-CoA dehydrogenase deficiencies, for the clinical significance of dehydrogenase impairments have become more strikingly evident.[1-3] An early harbinger of these interrelated chemical and clinical concerns may now be recognized in epidemeological, clinical, and chemical work related to the Jamaican vomiting sickness extending back more than 100 years.[4-7] The unripe fruit of the ackee tree,

(1) Tanaka, K.; Rosenberg, L. E. In *Metabolic Basis of Inherited Disease*, 5th ed.; Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., Brown, M. S., Eds.; McGraw-Hill: New York, 1983; pp 440–473.
(2) Gregersen, N. *Scand. J. Clin. Invest.* **1985**, *45*, *Suppl. 174*, 1–60.
(3) Kelly, D. P.; Whelan, A. J.; Ogden, M. L.; Alpers, R.; Zhang, Z.; Bellus, G.; Gregersen, N.; Dorland, L.; Strauss, A. W. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9236–9240.
(4) Bowrey, J. J. *Jamaica Gazette* **1887**, *10*, 481.
(5) Tanaka, K. In *Handbook of Clinical Neurology; Intoxications of the Nervous System*, Part 2; Vinken, P. J., Bruyn, G. W., Eds.; Elsevier/North Holland: Amsterdam, 1979; Vol. 37, pp 511–539.

0002-7863/92/1514-2245$03.00/0