oxidase and sulfite oxidase. There are substantial differences between the two model compound spectra in the beat region between k of 6.5 and 8.5 $Å^{-1}$ despite the basic structural similarity of the compounds. This points out the ability of the EXAFS method to easily distinguish the substitution of one ligand ($-SCH_3$ vs. $-N(CH_3)_2$) in the metal's coordination sphere. There is a striking similarity between the EXAFS of **1** and that of the oxidized form of sulfite oxidase.[2] While it cannot be concluded that the Mo environments are identical, there must be many common features.

Curve-fitting analysis of the EXAFS data for xanthine oxidase[2a] and sulfite oxidase[2b] suggests that, in their oxidized forms, both enzymes contain the MoO_2^{2+} unit. The average Mo=O distances, 1.71 Å, are the same for both proteins. Sulfur atoms are also present, at average distances of 2.42 and 2.54 Å for sulfite and xanthine oxidase, respectively. A more distant sulfur is also present at ~2.85 Å in both enzymes. The reason for the similarity of the EXAFS spectra for sulfite oxidase and **1** is clear—the Mo—O and both average Mo—S distances are very similar. The different appearance of the beat region of xanthine oxidase is simply a manifestation of the longer distance to the shorter sulfur ligands.

Comparison of the Mo—S distances in the enzymes with those of compounds **1** and **2** suggests configurational features about the Mo sites in the enzymes. The thiolate sulfurs in **1** and **2** are trans to each other and cis to the oxo groups with the Mo—thiolate distances all between 2.40 and 2.42 Å. The distances are consistent with other known structures containing thiolates cis to Mo=O.[8] Sulfite oxidase has similar Mo—S distances, but in xanthine oxidase a longer distance (2.54 Å) is observed. Since a pronounced trans effect is known to exist for Mo=O bonds, this longer distance may be characteristic of a thiolate sulfur trans to a Mo=O.

For every case in which structural information is available on Mo thiolate complexes (either from X-ray diffraction studies such as those reported herein or from ^1H NMR studies on several other complexes yet to be published[4]) the thiolate sulfurs are trans to each other and cis to the oxo groups. Attempts to synthesize complexes with three or four aliphatic thiolate groups about Mo(VI) have been unsuccessful, leading invariably to internal reduction of Mo(VI) to Mo(IV) or Mo(V). Three thiolate ligands on a MoO_2^{2+} core would require that at least one sulfur be trans to a Mo=O and that at least two thiolates be mutally cis. The strongly σ and π donating thiolate may thereby labilize the trans Mo=O, while the oxidation of two thiolates to a disulfide would be facilitated by their cis orientation on the Mo center. These factors may combine to allow facile reduction of Mo(VI) with concomitant loss of an oxo group. Should oxo activation be involved in enzyme-substrate reactions (either for Mo=O bond cleavage[11] and/or for proton transfer to oxygen),[11,12] the orientation of the thiolate ligands might be crucial for activating the Mo=O bond while mediating the redox potential of the Mo site.

These results further establish the validity of EXAFS analysis in the determination of accurate metal-ligand distances in molecules under noncrystalline conditions. The relevance of model complexes containing the MoO_2^{2+} core to the resting state structures of Mo in oxidized xanthine and sulfite oxidase has been defined by EXAFS. Further synthetic studies and EXAFS data analysis on the proteins in various states should lead to a more thorough understanding of their mechanisms of action on a molecular level.

Acknowledgments. This work was supported by the National Science Foundation through Grants PCM 17105 (to K.O.H.) and CHE 07026 (to E.I.S.). Synchrotron Radiation beam time was provided by the Stanford Synchrotron Radiation Laboratory supported by the National Science Foundation through Grant DMR-07692-A02 in cooperation with the Stanford Linear Accelerator Center and the U.S. Department of Energy.

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(1) Contribution 652 from the Charles Kettering Research Laboratories.
(2) (a) T. D. Tullius, D. M. Kurtz, Jr., S. D. Conradson, and K. O. Hodgson, *J. Am. Chem. Soc.*, following paper in this issue; (b) S. P. Cramer, H. B. Gray, and K. V. Ragagopalan, *ibid.*, preceding paper in this issue.
(3) The new ligands $(HSCH_2CH_2)_2NCH_2CH_2X$ where X = SCH_3, $N(CH_3)_2$, were prepared by reaction of $NH_2CH_2CH_2X$ with ethylene sulfide. The complexes were synthesized by the reaction of $MoO_2(acac)_2$ with the free ligand (1:1) in CH_3OH. Crystals of the yellow X = SCH_3 complex were grown either from a CH_2Cl_2 solution at 4 °C or from a 4:6:3 CH_3CN–CH_2Cl_2–C_6H_6 solution at room temperature. Two different crystal forms were observed. Crystals of the orange X = $N(CH_3)_2$ complex were grown from a solution of CH_2Cl_2–CH_2ClCH_2Cl at room temperature (acac = acetylacetonate). $MoO_2[(SCH_2CH_2)_2NCH_2CH_2SCH_3)]$ (**1**): ν(MoO) 910, 880 cm^{-1}. Anal. Calcd: C, 24.92; H, 4.48; N, 4.15. Found: C, 25.38; H, 4.52; N, 4.18. $MoO_2[(SCH_2CH_2)_2NCH_2CH_2N(CH_3)_2]$ (**2**): ν(MoO) 911, 882 cm^{-1}. Anal. Calcd: C, 28.74; H, 5.43; N, 8.38. Found: C, 28.48, H, 5.47, N, 8.43.
(4) E. I. Stiefel, J. L. Corbin, A. Elsberry, and N. Pariyadath, manuscript in preparation.
(5) S. P. Cramer, K. O. Hodgson, E. I. Steifel, and W. E. Newton, *J. Am. Chem. Soc.*, **100**, 2748 (1978).
(6) Crystal data: **1**, a = 7.235 Å, b = 7.717 Å, c = 24.527 Å, β = 119.86°, $P2_1/n$, Z = 4; **2**, a = 7.188 Å, b = 22.708 Å, c = 7.746 Å, $P2_12_12_1$, Z = 4. Data processing and structural solution methods are described in M. A. Bobrik, K. O. Hodgson, and R. H. Holm, *Inorg. Chem.*, **16**, 1851 (1977). The current unweighted R factors are 3.8% and 2.3% for **1** and **2**, respectively.
(7) Compound **1** was also observed to crystallize in a second crystal form which had two molecules per asymmetric unit. The structure of this crystal form was also determined by X-ray diffraction analysis and the basic structural features were the same as those for the crystal form reported in this communication. There were small differences in distances and angles and it was interesting to observe that the mean Mo—S (thioether) distance was 2.79 Å, a distance only 0.01 Å different from the EXAFS result. The two different unique Mo—S (thioether) distances in the asymmetric unit differed by 0.04 Å. Such a static disorder would result in a low number of atoms being predicted by the EXAFS analysis. Indeed, the number calculated from the EXAFS analysis was 0.5 rather than 1.0.[5] It is uncertain if the EXAFS data were collected on this crystal form, but it is noteworthy that the distances and calculated numbers of atoms are even more consistent with the X-ray result than for the form with only one molecule per asymmetric unit. Regardless of which form was used in the EXAFS analysis, the distinct similarities in the numbers compared with the crystallographic results remain.
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(13) Fellow of the Alfred B. Sloan Foundation, 1976–1978.
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The Molybdenum Site of Xanthine Oxidase. Structural Evidence from X-ray Absorption Spectroscopy

Sir:

We report here structural features of the molybdenum site of milk xanthine oxidase, as determined by analysis of the enzyme's X-ray absorption spectrum. Despite the prodigious amount of study which this metalloprotein has received over several decades,[1,2] this is the first definitive structural char-

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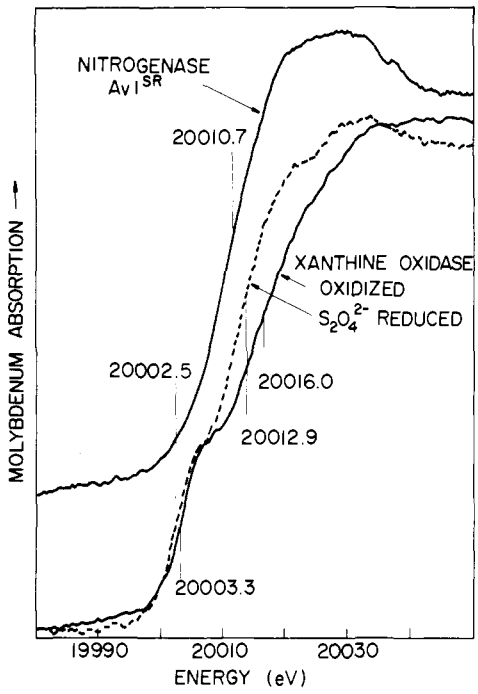

Figure 1. A comparison of the Mo K X-ray absorption edges for oxidized and dithionite reduced xanthine oxidase with that of the semireduced FeMo component of *Azotobacter vinelandii* nitrogenase. The energies of the inflection points as calculated numerically are shown. The edges of the xanthine oxidase bear striking resemblance to those of Mo complexes containing one or two Mo=O groups. Upon air oxidation, the nitrogenase edge also takes on a shape clearly indicative of Mo=O groups.

acterization of its molybdenum coordination environment.

Xanthine oxidase is the prototypical member of the class of molybdenum-containing hydroxylases.[1] It consists of two ~140 000-dalton subunits, each of which contains one Mo, two $Fe_2S_2(SR)_4$ clusters,[3] and one FAD. The enzyme catalyzes, among other reactions, the oxidation of xanthine to uric acid, which has been shown to take place at the molybdenum site. To complete the catalytic cycle, the electrons received by the protein from xanthine are used to reduce, for example, O_2 to H_2O_2. This half-reaction is carried out at the flavin site, remote from the Mo. The $Fe_2S_2(SR)_4$ groups are implicated in distribution of electrons over the catalytic sites. One of the Fe—S clusters has been shown to interact magnetically over a long distance with the Mo atom, by demonstration of splitting of the Mo(V) EPR signal by a reduced Fe species.[4]

Several components of the structure of the Mo site have been inferred, chiefly through interpretation of the Mo(V) EPR signals observed from various enzyme states (catalytically active, inactive, and inhibited). The EPR g values, when compared with model spectra, were suggestive of some sulfur ligation.[5,6] Most evidence is consistent with the Mo cycling through the IV, V, and VI states. From the known structural chemistry of high-valent Mo and the possibility of a metal to ligand "oxo" transfer in the catalytic cycle, the presence of terminal oxo groups (Mo=O) appeared reasonable but by no means certain. Dimeric Mo species have been considered, but appear less likely because of the equivalence of the two protein subunits and the two separate sets of catalytic machinery. Splitting of most of the Mo(V) EPR signals by a proton is observed, and this has been explained by assuming the presence of a proton associated with a ligand of the Mo (and not a Mo hydride).[6] Finally, the protein as isolated is usually a mixture of catalytically active and inactive forms. From studies on cyanide inactivation, it has been suggested that conversion of active to inactive enzyme is due to loss of either a persulfide[7a] or a cysteine[7b] sulfur atom. Whether or not this sulfur atom is a ligand of the Mo is still open to question.

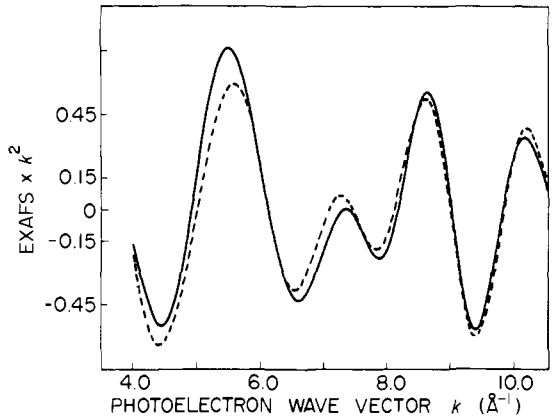

Figure 2. Fourier filtered (k = 4–10.5, R = 0.3–3.0 Å) oxidized xanthine oxidase data (—). The beat clearly indicates the presence of at least two different Mo—L distances. The best three-wave fit to the data (O,S,S′ waves) (---) results in the distances and coordination numbers given in Table I. Fit range: 4–10.5 Å^{-1}, k^6 weighting.

The technique of X-ray absorption spectroscopy has proven to be a powerful method for investigating the structure of specific metal sites in metalloproteins under noncrystalline conditions.[8-14] Encouraged by our success in elucidating the Mo coordination environment in nitrogenase,[8] and in the FeMo cofactor,[9] we have extended these studies to other Mo proteins. Our method of analysis of the extended X-ray absorption fine structure (EXAFS) has been described in detail[15-16] and has been shown to give metal–ligand bond distances to an accuracy of typically ±0.02 Å. Within certain limits, the method also allows determination of the number and type of ligands, even in complex, multishell structures.[15-17]

Xanthine oxidase was isolated from unpasteurized buttermilk by the procedure of Massey et al.[18] Activity to flavin ratios at 25 °C were determined as described[18] for samples both before and after the X-ray absorption experiments and found to be identical within the range 121 ± 5. The reduced enzyme was prepared by anaerobic reduction with a 30-fold molar excess of $Na_2S_2O_4$ over enzyme flavin and showed absorbance changes identical with those reported in the literature.[18] The buffer used in these experiments was 0.1 M Tris-acetate (pH 8.5) containing 1 mM EDTA. X-ray absorption spectra were recorded at 25 °C on Stanford Synchrotron Radiation Laboratory line 1 using methods described previously.[15,16]

The Mo K absorption edges of oxidized and dithionite reduced xanthine oxidase are shown in Figure 1. Unlike the smooth edge characteristic of the nitrogenase FeMo component, both xanthine oxidase edges exhibit two distinct inflection points. The presence and intensity of the first inflection at ~20 003 eV (assigned to a 1s → 4d bound-state transition)[19] argues strongly for at least one and more likely two terminal oxo groups bound to Mo in the oxidized form of the protein.[8,10] Based on the absorption edges of numerous other Mo complexes with biologically relevant ligands, the position of the second inflection at 20 016 eV also suggests the presence of oxo groups. Upon reduction, the higher energy inflection is seen to move by ~3.1 eV to lower energy. This shift could result from either a formal two-electron reduction of the Mo (Mo(VI) to Mo(IV)) while maintaining the same ligation, or the loss of one oxo group, or a combination of both.[19] For example, the edge of the ionic $MoCl_3$ is lower by 3.9 eV than that of $MoCl_5$,[8] and the edge of MoO_3(dien) is ~1.6 eV lower than that of MoO_4^{2-}. Only the Mo(IV) or the Mo(VI) oxidation states are consistent with the absence of a Mo EPR signal in the reduced enzyme. Considering the relatively low energy value for the second inflection, the edge results are then most consistent with reduced xanthine oxidase containing Mo(IV).

Table I. Xanthine Oxidase Fitting Results[a]

shells	F[b]	O	S	S′
O	1.83	1.71 [1.8]		
O,S	0.82	1.71 [1.5]	2.52 [2.0]	
O,S,S'_{short}	0.80	1.72 [1.5]	2.52 [2.0]	2.16 [0.2]
O,S,S'_{long}	0.54	1.71 [1.5]	2.54 [2.1]	2.84 [1.1]

[a] Values in brackets are calculated numbers of atoms, fits weighted $k^6(\text{data} - \text{fit})^2$. [b] $F = [\Sigma k^6(\text{data} - \text{fit})^2/N]^{1/2}$.

To gain a more quantitative understanding of the structure of the Mo site, we have analyzed the Mo EXAFS of oxidized xanthine oxidase with our curve-fitting techniques. The most obvious feature of the EXAFS data (Figure 2) is the presence of a "beat" in the amplitude envelope, which is direct evidence of at least two different Mo—L distances. The best two-wave fit determined 1.5 oxygen atoms at 1.71 Å, and 2.0 sulfur atoms at 2.54 Å. Including another sulfur atom at a longer distance significantly improved the quality of the fit, especially in the beat region. This three-wave fit is compared with the data in Figure 2 and the numerical results of the fits are summarized in Table I.

Fits using several other combinations of ligands and distances were tried, none of which gave reasonable results. For example, the oxo (short oxygen) wave was absolutely required, and no evidence was found for the presence of terminal sulfur alone or in combination with terminal oxygen. No other atom type but sulfur would satisfactorily reproduce the beat when combined with the oxo wave. The identity of the longer sulfur was also unique, and it could not be replaced by a sulfur at a shorter distance (see Table I for the results of one such fit).

The distances and coordination numbers derived from the best three-wave fit lead to a model for the oxidized state of xanthine oxidase which is in accord with known Mo structural chemistry.[6] The Mo—O distance of 1.71 Å is similar to that found for Mo complexes which have several terminal oxo groups,[17] and is not consistent with a bridging oxo group distance. The intermediate distance sulfurs are most likely thiolates, although the distances are slightly longer (~0.1 Å) than observed for thiolates in other MoO_2L_4 complexes.[17] Although the EXAFS contains no information about the angular disposition of the ligands, this longer distance suggests that these sulfurs may be trans to the Mo=O groups (which are themselves normally cis in Mo(VI) complexes)[17] owing to the well-known trans effect of Mo=O. The longer sulfur ligand could be a thioether, consistent with the structure reported in the accompanying communication.[17] An alternative formulation for this sulfur could be as the second sulfur of a persulfide. Unfortunately, no examples of this type of coordination yet exist in Mo complexes.

Persulfide ligation logically raises the question of the nature of the cyanide inactivation of xanthine oxidase. As our enzyme was not purified of the "desulpho" form,[1] the Mo site could reflect inhomogeneity owing to the presence of both forms (if indeed the inactivation involves the primary coordination environment of the Mo[7]). This could be a reason for our finding a nonintegral number of oxo groups. It is not possible, given the present data, to make any further conclusions regarding this question. However, the clear ability of EXAFS to distinguish the presence or absence of a single sulfur, even at 2.84 Å,[17] makes such experiments quite feasible.

Because of the preliminary nature of these experiments, the data were good only over a limited range. If there were another scattering atom of a low atomic number between 2 and 2.5 Å, it would be difficult to observe in our present data. An amine nitrogen has been suggested[6] as the ligand with the acidic proton which splits the Mo(V) EPR signal.

The geometry of the Mo site in xanthine oxidase contrasts with that determined by EXAFS for sulfite oxidase,[20] even though the ligand composition is basically the same. Sulfite oxidase has two or three sulfurs at a more usual 2.42-Å average distance, suggesting that they are not all trans to oxo groups. This observation suggests that the protein is able to tailor its active site for the type of chemistry to be performed by defining the geometric arrangement of the metal's ligands. For example, if xanthine oxidase needs (as a part of its catalytic mechanism) to lose a thiolate sulfur from the Mo coordination sphere, positioning a thiolate trans to an oxo could effect such a labilization. In fact, the ease of loss of sulfur from the Mo site upon treatment with CN^- could be a result of this trans labilization of a thiolate sulfur.

Together with the recently reported dimeric nature of the Fe-S centers in xanthine oxidase,[3] the results reported herein point toward the complete structural characterization of the metal sites in this enzyme. Much less is known about the relative spatial arrangement of the sites and complete details may well await a single-crystal structure determination. It is important to note that these EXAFS results have been obtained in a relatively short period of time and that the EXAFS method is well suited to carry out further experiments on xanthine oxidase in solution. Studies of the enzyme in its cyanolyzed "desulpho" form, in its various redox states, and in its inhibited form in the presence of substrate analogues will define even further the role of Mo in the enzymatic function of xanthine oxidase. Finally, these EXAFS studies (and the analogous studies on sulfite oxidase[20]) have elucidated a new type of coordination environment for Mo in a metalloprotein which is distinctly different from that observed in nitrogenase.[8-10]

Acknowledgments. We thank Steve Cramer for helpful discussions. This work was supported by the National Science Foundation through Grant PCM-17105. Synchrotron radiation beam time was provided by the Stanford Synchrotron Radiation Laboratory supported by National Science Foundation Grant DMR-07692-A02 in cooperation with the Stanford Linear Accelerator Center and the U.S. Department of Energy.

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(19) Representative Mo K X-ray absorption edges can be found in ref 8 and 9. For Mo in a covalent environment, transitions into optical levels below the continuum (molecular orbitals comprised of ligand and metal contributions) give rise to the structure observed superimposed on the overall absorption edge. Most striking is the transition observed at ~20 003 eV. Preliminary SCF-Xα calculations identify an allowed transition of A_1 (1s) to T_2 (mainly metal 4d and ligand p) symmetry in MoO_4^{2-}, $MoO_2S_2^{2-}$, and MoS_4^{2-} which lies below the continuum and whose position is quite insensitive to the

number of oxo vs. sulfur ligands. Quantitative aspects of these effects will be published in a subsequent paper.
(20) S. P. Cramer, H. B. Gray and K. V. Rajagopalan, *J. Am. Chem. Soc.*, accompanying paper in this issue.
(21) Recipient of a National Institutes of Health Postdoctoral Award No. GM 0657-02.
(22) Fellow of the Alfred P. Sloan Foundation, 1976-1978.

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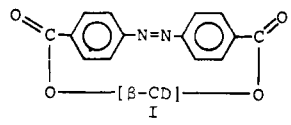
Photocontrol of Binding Ability of Capped Cyclodextrin

Sir:

Photosensitive systems are ubiquitous in nature. Many of the life processes of plants are regulated by the effect of light on the phytochrome system. The process of vision falls into the category of fundamental animal responses. These physiological changes are believed to be linked with light-induced structural changes.[1] The activity of biologically active macromolecules can be regulated by low-molecular-weight photochromic molecules capable of assuming (at least) two states.[1] As one approach to mimic such biological systems, we have examined the photoresponsive behavior of polypeptides containing azobenzene moieties in their side chains and have found the existence of light-induced conformational changes.[2] An additional refinement in our studies is the photoregulation of functions such as substrate binding and catalytic activity. From this strategy, we prepared azobenzene-capped β-cyclodextrin I to regulate the binding ability of β-cyclodextrin (β-CD) by light.[3] The parent β-CD itself represents a good enzyme model because of its ability to bind substrates into its cavity in aqueous solution. Compound I is expected to act as a photoregulated "switch" since the cap azobenzene undergoes cis-trans isomerization by photoirradiation, and the reversion of the cis isomer back to the trans takes place in the dark.[4] We now report (i) the preparation of I; (ii) changes in the circular dichroism spectra of I on addition of guest molecules; (iii) photoregulation of the binding ability of I, and (iv) the presence of 1:2 host-guest complexes[5] and photoregulation of their formation.



I

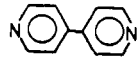
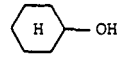
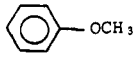
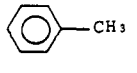
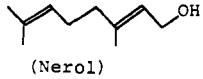
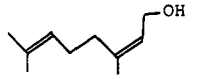
Compound I was obtained by condensation of 4,4′-bis-(chlorocarbonyl)azobenzene with β-CD in pyridine (20% yield). Recrystallization from water and Sephadex G-15 gel filtration[6] gave a pure sample of I.[7]

Figure 1 shows the circular dichroism spectra of a 5×10^{-5} M solution of I in water (Tris buffer, pH 7.2) before and after photoirradiation, alone and in the presence of excess cyclohexanol. Compound I presents an induced circular dichroism band in the azobenzene π-π^* region (355 nm) before irradiation, whereas it shows another band in the azobenzene n-π^* region (445 nm) after irradiation. Both induced circular dichroism bands nearly vanish on addition of guest molecules in large excess. This observation might reflect the transformation of β-CD residue from a "tense" conformation to a "relaxed" one upon inclusion of guest molecules as was reported by Saenger et al. for α-CD.[8] Formation constants K (or dissociation constants K_d) of *trans*-I and *cis*-I were obtained from the circular dichroism spectra (intensities at 355 and 445 nm were used for *trans*-I and *cis*-I, respectively) using the formula

$$K = \frac{\theta_I - \theta_x}{(\theta_x - \theta_s)\left[C_s - C_I \dfrac{\theta_I - \theta_x}{\theta_I - \theta_s}\right]}$$

which was reported by Mack et al. for 1:1 host/guest complex formation[9] where θ = molar ellipticity, θ_x for sample, θ_I for I alone, θ_s for highest substrate excess, C_I = total I concentration, and C_s = total substrate concentration. It was found that there are some cases which do not follow the formula but proceed according to the equation[10]

Table I. Dissociation Constants for Complexes of *trans*-I and *cis*-I with Various Substrates[a]

guest	host	$K_d(K_{d1})$, M	K_{d2}, M	$\frac{K_d(trans\text{-I})}{K_d(cis\text{-I})}$	$\frac{K_d(trans\text{-I or } cis\text{-I})}{K_d(\beta\text{-CD})}$
	β-CD	7.3×10^{-3}			
	trans-I	no complex formed			
	cis-I	2.2×10^{-3}			0.30
	β-CD	2.5×10^{-3} [b]			
	trans-I	3.9×10^{-3}		2.0	1.6
	cis-I	2.0×10^{-3}	1.6×10^{-3}		0.80
	β-CD	1.9×10^{-2}			
	trans-I	5.0×10^{-2}		3.8	2.6
	cis-I	1.3×10^{-2}	7.8×10^{-3}		0.68
	β-CD	1.5×10^{-1}			
	trans-I	4.9×10^{-2}		4.5	0.33
	cis-I	1.1×10^{-2}	1.2×10^{-2}		0.073
 (Nerol)	β-CD	1.7×10^{-3}			
	trans-I	3.2×10^{-3}		3.8	1.9
	cis-I	8.5×10^{-4}	1.5×10^{-3}		0.50
 (Geraniol)	β-CD	1.5×10^{-3}			
	trans-I	3.6×10^{-3}		8.6	2.4
	cis-I	4.2×10^{-4}	2.9×10^{-3}		0.28

[a] In 0.05 M Tris buffer (pH 7.2) at 25 °C. Substrates were added as CH_3CN solutions (total content of CH_3CN is smaller than 1% (v/v)). [b] Reported value, 2.0×10^{-3} M, in 0.05 M borate buffer (pH 10.0) at 25 °C.[3d]

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