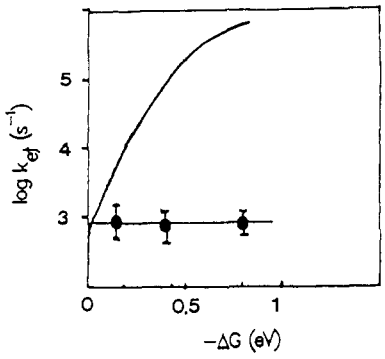

Figure 1. Plot of b_5 K_{et} vs $-\Delta G$ for the intra complex electron transfer between b_2 and (M)cytc where M = Fe(III) ^{3}(Zn*), ^{3}(porph*). The top solid line shows the dependence predicted by Marcus theory (for $\lambda \sim 1$ V, as e.g., in cytochrome c/cytochrome b_5): conditions pH 7 (5 mM Pi), 25 °C.

and Ratner have recently argued that for proteins, redox linked configurational changes may well be rate limiting.[15] In such cases, the familiar dependence of rate on reaction free energy would not hold. We now present evidence for such "conformational gating"[15] in the reaction of cytochrome c (c) with cytochrome b_2 (b_2) (yeast lactate dehydrogenase). This system is of special interest for several reasons. First the b_2/c system has been the subject of long standing elegant studies by Labeyrie and co-workers.[12,16] Second, the structure of b_2 has recently been solved by Matthews,[17] and the prospect exists of obtaining a detailed structure for the complex.[16] Finally, well characterized metal substituted cytochrome c derivatives are available, so that ΔG can be easily varied in the c/b_2 complex.[9] Cytochrome c and the derivatives H_2 porphyrin cyctochrome c (porph c) and Zn(II) cytochrome c (Znc) were prepared and purified as previously described.[9,15] Cytochrome b_2 was purified from *Saccaromyces cervisiae*, as described previously.[18] Electron transfer within the preformed $Fe^{II}c/Fe^{III}b_2$ complex has been previously studied in two labs. Capelliere Blandin used stopped flow techniques to measure a rate constant of 380 s^{-1} at 5 °C between *H. anomola* cytochrome b_2^{II}/cytochrome c^{III}. With use of the reported activation energy (3.3 Kcal M^{-1}), the rate constant at 25 °C would be 570 s^{-1}. For horse cytochrome c, the rate is reported to decrease about fourfold.[11b]

In the present work we have used the lumiflavin chemistry pioneered by Tollin and Cusanovich[9c] to photochemically reduce cytb_2 and follow electron transfer within a preformed (horse) c/b_2 complex and find a rate constant of 200 ± 80 s^{-1} under our conditions (25 °C, pH 7, 5 mMPi) in reasonable agreement with previous work. Thus, while the rate of intracomplex electron transfer is sensitive to the primary sequence of the protein and perhaps to specific solution conditions as well, a range of rates of 600 ± 300 s^{-1} encompass all these variations for the native $Fe^{II}b_2/Fe^{III}c$ reaction. In order to better characterize the parameters which control electron transfer in the cyt$c$/cytb_2 system, we have utilized redox photoactive derivatives[9,10] (e.g., Znc) which provide a range of reaction free energies: for the porphc/b_2 complex $\Delta G \cong -0.4$ V, for the Znc/b_2 complex $\Delta G \cong 0.8$ V. As discussed in detail elsewhere,[9-11] the Znc and porph$c$ derivatives are essentially isostructural with the native cytochrome c. They form strong, specific complexes with cytochrome b_2, as shown by fluorescence energy transfer experiments.[11] Furthermore, both Znc and porph$c$ act as strong competitive inhibitors in steady-state enzyme assays of the lactate/b_2/Fec reaction:[11a] for Znc we find $K_I \cong K_m \cong 10$ μM. The available evidence thus suggests that Fec, Znc, and porph$c$ all form equivalent complexes with cytochrome b_2 and the electron-transfer rates among these complexes should proceed by similar mechanisms. Both theory and previous experimental results on other protein complexes suggest that the large change in ΔG between b_2/Fec and b_2/Znc should result in correspondingly large differences in electron-transfer rates. To our surprise, and in contrast to other protein systems,[7,9,10] reaction rates for these c/b_2 complexes are essentially *independent* of ΔG over this wide range (Figure 1: k_{Zn/b_2} = 600 (± 200) s^{-1}, k_{porph/b_2} = 700 (±100) s^{-1})!

We believe these results offer compelling support for the importance of conformational control of reaction rates, in a complex formed between physiological protein reactants. In the "gating" mechanism proposed by Hoffman and Ratner,[16] the overall reaction rate can be controlled by the rate of formation of "redox active" conformation within the complex, and this need not depend on those factors like free energy and electronic coupling of strengths which normally govern electron transfer rates. The current results are fully consistent with this theory.[20] We note that the existence of such conformational "upper limits" to reaction may exist even when a normal dependence of rate or free energy is observed, as in the cytochrome c/cytochrome b_5 complex,[9] which could lead to lower overall rates than would be expected in the absence of such conformational barriers. The source of such conformational barriers remains unclear. We are currently undertaking studies by using site directed mutagenesis to help clarify this and related questions.

Acknowledgment. This work was supported by the NIH (GM33881) and in part by the NSF (CHE8303896). We gratefully acknowledge helpful discussions with Brian Hoffman, Mike Cusanovich, Chantal Cappelliere Blandin, and Harry Gray.

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(21) The large quoted uncertainties (70%) reflect the range of values found with independent preparations of b_2 from different yeast sources. Multiple determinations on single samples were precise to <5%. Possible mechanisms for the rate increase on binding to b_2 include enhanced nonradiative decay within the complex and energy transfer. Both these mechanisms are doubtful, since Znc/$Fe^{II}b_2$ shows an *increased* lifetime for the ^{3}(Znc). Energy transfer is further ruled out by the fact that the rate constants for (Znc/porph$c$) χ10 are based on the difference in spectral overlap with b_2 of these derivatives.

Trinitrosyl Species on Supported Iron Catalysts

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Received July 27, 1987

Nitric oxide has frequently been used as a probe molecule for assessing the adsorbed state of catalytically active metallic species on the surface or in the bulk of oxides and zeolites.[1] In the case of cobalt[2-9] and iron,[10-13] a pair of infrared bands near 1900 cm^{-1}

†On leave from Laboratoire de Spectroscopie, Faculté des Sciences, Limoges, France.
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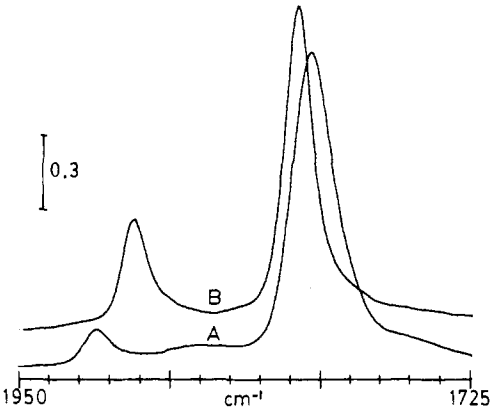

Figure 1. Infrared spectra of $Fe(CO)_2(NO)_2$ (curve A) and $Co(CO)_3NO$ (curve B) adsorbed on zeolite-Y in the presence of excess (10–100-fold) gaseous NO.

[weak] and *1800* cm^{-1} [strong] have been consistently assigned to the symmetric and antisymmetric stretching modes, respectively, of a dinitrosyl species attached to either metal. In this communication we show that although such an assignment is correct in the case of cobalt, for iron these bands should probably be attributed to a trinitrosyl species.

Figure 1 shows the infrared spectrum observed following the adsorption of $Co(CO)_3NO$ or $Fe(CO)_2(NO)_2$ in the presence of excess (10–100-fold) gaseous NO on Na-zeolite-Y which had been previously activated in a vacuum at 450 °C.[14] The spectra in both cases were recorded about 1 h after the initial adsorption during which time the CO bands disappeared. Isotopic labeling experiments with C-13 and N-15 showed that the pair of bands were due to NO species.[15] For Fe, the wavenumbers are 1914 and 1806 cm^{-1}, respectively, having a half width of 22 cm^{-1}, whereas for Co they are at 1892 and 1810 cm^{-1}, respectively,[9] having a half-width of 15 cm^{-1}. A similar pair of bands at 1880 and 1800 cm^{-1} (half-width 40–45 cm^{-1}) has also been observed by us under the same conditions for deposition of $Co(CO)_3NO$ on Al_2O_3[8] which was shown by isotopic labeling to be due to a dinitrosyl species. Dinitrosyl species have also been formed from NO and a Co^{2+} exchanged zeolite-Y.[6,7]

Once the Fe or Co species responsible for the 1900/1800 cm^{-1} bands has formed on NaY it rapidly exchanged with ^{15}NO, and we have used the labeling technique to determine whether the iron species is also a dinitrosyl. Figure 2 shows that the predicted three components for a dinitrosyl were generated in the case of the cobalt compound. For the iron compound, quite unexpectedly the high wavenumber symmetric band split into four components for partial exchange (Figure 3), and in the case of 50% isotopic exchange (confirmation of the gas phase by mass spectrometry), the relative intensities were approximately in the ratio 1:3:3:1. This shows that a trinitrosyl species was present, the four bands being due to the four possible $^{14}NO/^{15}NO$ combinations from a $Fe(NO)_3$ fragment initially in a C_{3v} environment.[16]

In the region of the low wavenumber band at 1806 cm^{-1} we also observed four poorly resolved components for partial isotopic exchange (not shown). These bands for a C_{3v} $Fe(^{14}NO)_3$ and $Fe(^{15}NO)_3$ at 1806 and 1770 cm^{-1}, respectively, would be doubly degenerate, and degradation of this symmetry to C_s as in $Fe(^{14}NO)_2{}^{15}NO$ and $Fe^{14}NO(^{15}NO)_2$ would cause a splitting of this mode into two bands for each of these isotopomers. Therefore, a total of six bands would be expected in this spectral region for all four mixed isotopic species. Given that the half-width of each component would be about 22 cm^{-1} our failure to resolve all of these bands in this spectral region was not unexpected.[17]

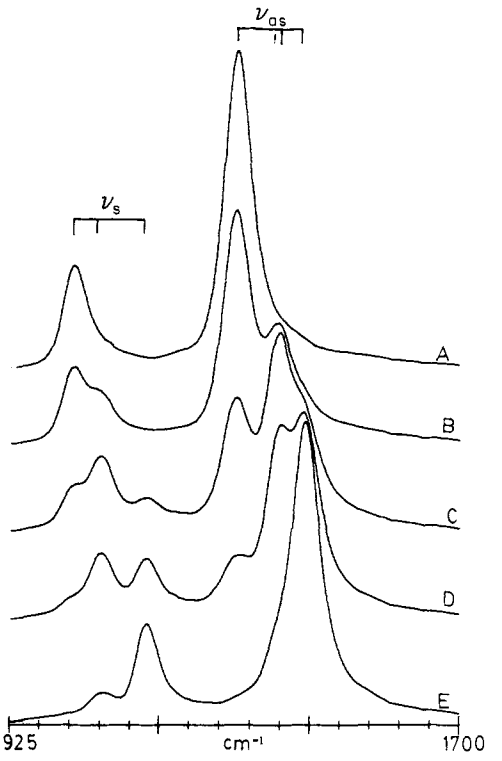

Figure 2. Infrared spectra of $Co(CO)_3NO$ on zeolite-Y (curve A) and subsequent spectra (curves B to E) showing the effect of progressively increasing exchange with nitrogen-15 up to about 90% exchange in curve E. Vertical lines indicate the approximate positions of the three peaks for both the ν_s and ν_{as} stretching modes of a dinitrosyl species of isotopic composition $^{14}NO^{14}NO$, $^{14}NO^{15}NO$, and $^{15}NO^{15}NO$.

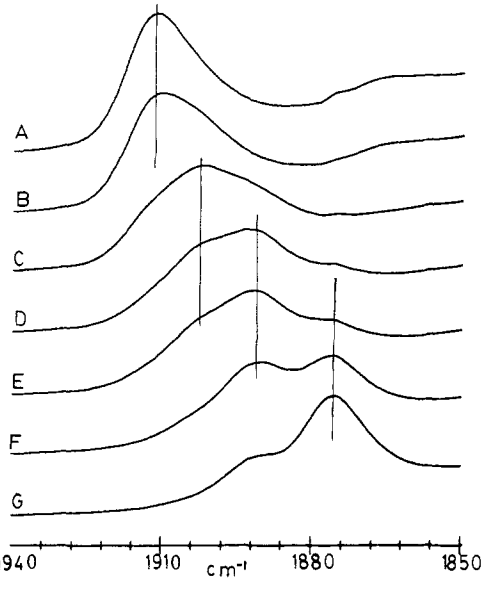

Figure 3. Infrared spectra of the high wavenumber symmetric NO stretching band of $Fe(CO)_2(NO)_2$ adsorbed on zeolite-Y (curve A) and subsequent spectra (curves B to F) showing the effect of progressively increasing exchange with nitrogen-15 up to about 90% exchange. Vertical lines show the approximate positions of the positions of the four anticipated bands for various mixed isotopic trinitrosyl species (see text).

(14) See ref 8 and 9 for details concerning the deposition of $Co(CO)_3NO$: similar conditions were used for $Fe(CO)_2(NO)_2$. Spectra were recorded by using a Bomem DA3.02 FTIR.

(15) The weak absorption at 1860 cm^{-1} in Figure 1A increases at the expense of the pair at 1914/1806 cm^{-1} upon admission of a small amount of O_2 and belongs to a different species, likely a mononitrosyl on Fe(III), see ref 10.

(16) For an example of a structurally characterized $Mn(NO)_3$ complex in a C_{3v} environment, see: Wilson, R. D.; Bau, R. *J. Organomet. Chem.* **1980**, *191*, 123.

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The species responsible for the 1914/1806 cm^{-1} bands is likely to be of the same nature as those observed from the interaction of NO with Fe^{2+}–Y[10,11] or with Fe(II) on SiO_2.[12,13] They have been assigned previously to a surface dinitrosyl, but the results here imply that a reinterpretation of these earlier studies might be appropriate. The unique feature of the present work which has allowed us to distinguish so clearly between the di- and trinitrosyl has been (1) the deposition technique used which permitted the formation of only one NO–Fe species having intense bands in the 1800–1920-cm^{-1} region and (2) the extremely narrow half-width of the infrared bands which has permitted us for the first time to just resolve the four expected components of a mixed isotopic trinitrosyl surface species. Most importantly, the present results establish that on supported iron surfaces, three rather than two coordination sites can in principle be available for catalysis.

Acknowledgment. We are grateful to N.S.E.R.C. of Canada for financial support.

3-Halovinylglycines. Efficient Irreversible Inhibitors of *E. coli* Alanine Racemase

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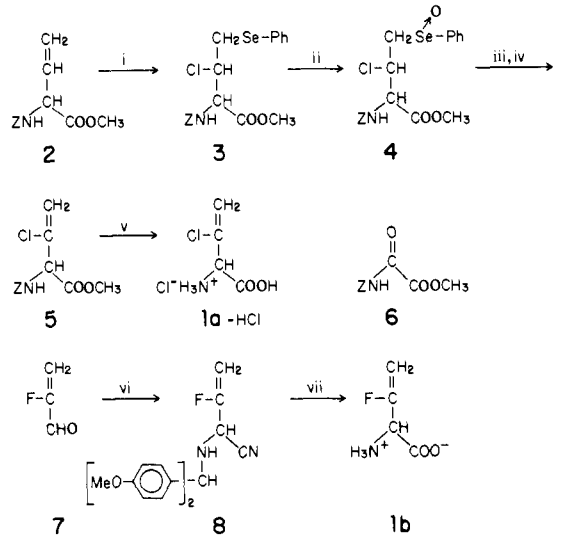
Received September 22, 1986

We wish to report that 3-halovinylglycines **1** are a new class of potent mechanism-based irreversible inactivators of alanine racemase. Our results with *Escherichia coli B* alanine racemase indicate that nearly every catalytic event results in irreversible inactivation of the enzyme. In contrast, mechanism-based inactivation by previously studied β-substituted alanines is much less efficient, requiring approximately 800 turnovers with this enzyme to produce one irreversible event.[1]

Alanine racemases are prime targets for design of antibiotics because they are unique to bacteria and are essential for production of D-alanine, required in cell wall biosynthesis.[2] The utility of this approach has been established by 3-fluoro-D-alanine, which is a potent, broad spectrum, orally active antibiotic.[3] However, safety issues unrelated to its mechanism of action have hindered the clinical application of 3-fluoro-D-alanine. Consequently, the search has continued for new agents with this mechanism of action. We now describe studies with 3-halovinylglycines which are particularly effective inhibitors of bacterial alanine racemase.

Our syntheses of 3-chlorovinylglycine [2-amino-3-chlorobutenoic acid, **1a**] and 3-fluorovinylglycine [2-amino-3-fluorobutenoic acid, **1b**] are outlined in Scheme I.[4] D-, L-, and DL-3-chlorovinylglycine were obtained from the corresponding *N*-(benzyloxycarbonyl)-vinylglycine methyl ester (**2**) which in turn was derived from the corresponding D-, L-, or DL-methionine.[5] Extension of this methodology to 3-fluorovinylglycine was not successful, and it was

Scheme I[a]



[a] (i) Ph–SeCl, CH_3CN, 0–25 °C, 2 h;[8] (ii) O_3, CCl_4, −20 °C; (iii) CCl_4, 1 equiv of pyridine, reflux; (iv) silicagel chromatography, elution with EtOAc: hexanes = 1:4, 15–20% yield from **2**;[9] (v) 6 N HCl reflux 80 min, 80–90% yield; (vi) 4,4′-dimethoxybenzhydrylamine, 4 Å molecular sieves, CH_2Cl_2 followed by TMS–CN, 25 °C;[7] (vii) 6 N HCl, reflux 2 h followed by Dowex (H^+) chromatography, elution with 3% pyridine in water, 25–30% yield from **7**.

prepared from 2-fluoroacrolein (**7**)[6] by a modified Strecker synthesis.[7]

Incubation of homogeneous *E. coli B* alanine racemase[10] with D-chlorovinylglycine, L-chlorovinylglycine, or DL-fluorovinylglycine results in irreversible inactivation of the enzyme as demonstrated by the inability of the enzyme to regain catalytic activity after prolonged dialysis. The inactivation kinetics are characterized by rapid, pseudo-first-order irreversible inhibition of 70% of the enzyme. The second-order rate constant that describes this initial inactivation for D-chlorovinylglycine (122 ± 14 M^{-1} s^{-1}) is comparable to the corresponding rate constant for 3-fluoro-D-alanine (93 ± 13 M^{-1} s^{-1}). Irreversible inhibition of the remaining enzyme is described by a first-order rate constant that is independent of inhibitor concentration, halogen, and stereochemistry ($1.2 \pm 0.4 \times 10^{-4}$ s^{-1}). A complete description of the inactivation kinetics has been obtained and will be published later.

Partition ratios were determined in parallel experiments for 3-fluoro-D-alanine and D-chlorovinylglycine (Figure 1). The value of the *x*-intercept represents the inhibitor concentration required to inactivate irreversibly all of the enzyme in the incubation mixture. As can be appreciated from a comparison of the two plots, the *x*-intercept for D-chlorovinylglycine is approximately 300-fold lower than that for 3-fluoro-D-alanine. Assuming a partition ratio of 800 for 3-fluoro-D-alanine,[1] the results from three independent experiments indicate that 2.1 ± 0.6 molecules of D-chlorovinylglycine are required to inactivate 1 molecule of enzyme. The partition ratio that is measured for L-chlorovinylglycine

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(7) Procedure of Greenlee, W. J. *J. Org. Chem.* **1984**, *49*, 2632. R. D. Hoffsommer in our laboratory had previously obtained **1b** in trace acounts from **7** via 3-fluorovinylglycolic acid utilizing Baldwin's route to racemic vinylglycine (Baldwin, J. E.; Haber, S. B.; Hoskins, C.; Kruse, L. I. *J. Org. Chem.* **1977**, *42*, 1239).
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(9) *N*-Benzyloxycarbamoyl methyl oxamate **6**, mp 89–90°, a byproduct of the ozonization of **3**, is also produced on CrO_3 based oxidation of Z-Ser-OMe and Z-Thr-OMe (Statchulsky, A. V. *Tetrahedron Lett.* **1982**, *23*, 3789). Ozone oxidation at the α-carbon of **4**, analogous to the O_3 oxidation of acetals (DesLongchamps, P.; Moreau, C. *Can. J. Chem.* **1971**, *49*, 2465), followed by fragmentation would yield **6**.
(10) The enzyme used in these studies was purified from *E. coli* B (MB 1967) that was grown in a medium containing L-alanine as the sole carbon source. The enzyme was purified to homogeneity in 35% yield by using a procedure that will be reported separately.

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