# Steric and Hydrophobic Effects in Alkyl Isocyanide Binding to *Rhodospirillum molischianum* Cytochrome *c'* †

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*Received March 10, 1988; Revised Manuscript Received October 3, 1988*

ABSTRACT: Equilibrium constants for the binding of a series of alkyl isocyanides to ferrous cytochrome *c'* from *Rhodospirillum molischianum* have been measured spectrophotometrically. The equilibrium constants range from 3.3 $M^{-1}$ to $2.6 \times 10^2$ $M^{-1}$ and follow the order methyl > ethyl < *n*-propyl < *tert*-butyl < *n*-butyl < amyl < cyclohexyl < *n*-hexyl. The decrease in equilibrium constant from methyl to ethyl isocyanide provides evidence for a steric interaction between the ligand and the protein. The increase in equilibrium constant from ethyl to *n*-hexyl isocyanide is accounted for by a favorable partitioning of the ligand into a hydrophobic heme coordination site. The effect of steric interactions on the differences in the binding constants has been further evaluated by comparing the alkyl isocyanide and CO binding constants for the ferrous cytochrome *c'* to those of a sterically unconstrained model heme complex in a detergent micelle. The results indicate that the heme coordination site of the ferrous cytochrome *c'* is severely sterically hindered, similar to that of the reported crystal structure of *Rs. molischianum* ferric cytochrome *c'*.

The cytochromes *c'* represent a unique family of *c*-type cytochromes found in a wide variety of photosynthetic and denitrifying bacteria (Bartsch, 1978). With one exception, the native protein is usually isolated as a dimer of identical subunits having a molecular weight of 14000 per subunit. Each monomer incorporates a covalently bound protoheme IX prosthetic group. These proteins have a heme binding sequence pattern similar to the class of low-spin mitochondrial *c*-type cytochromes but have optical absorption spectra and CO binding properties resembling high-spin hemoglobins and myoglobins (Bartsch, 1978). X-ray crystallographic studies of *Rhodospirillum* (*Rs.*) *molischianum* cytochrome *c'* and spectroscopic studies of this and other cytochromes *c'* indicate that the heme iron is pentacoordinate, with a histidyl imidazole group providing the single axial ligand to the heme iron (Weber et al., 1980, 1981; Finzel et al., 1985).

In contrast to hemoglobin and myoglobin, however, earlier reports indicated that ligand binding to these proteins was limited to CO and NO (Taniguchi & Kamen, 1963), and only carbon monoxide has been the subject of in-depth studies (Cusanovich & Gibson, 1973; Doyle et al., 1985, 1986, 1987). The crystal structure analysis of ferric cytochrome *c'* from *Rs. molischianum* indicated that the ligand binding properties may be explained in terms of a distal heme surface surrounded by hydrophobic amino acid residues that are oriented so as to restrict access of exogenous ligands to the sixth heme iron coordination site (Weber et al., 1981; Finzel et al., 1985). Studies from our laboratory have more recently demonstrated that ethyl isocyanide will bind to ferrous cytochromes *c'* from *Chromatium* (*C.*) *vinosum* (Kassner et al., 1983), *Rhodopseudomonas* (*Rh.*) *sphaeroides*, *Rhodopseudomonas* (*Rh.*) *palustris*, and *Rhodospirillum* (*Rs.*) *rubrum* and exhibit more than a 1000-fold difference in equilibrium constants and more than a 2-fold difference in extinction coefficients (Rubinow & Kassner, 1984). The differences in binding properties have been suggested to result from sterically hindered ligation to the heme iron.

Alkyl isocyanides with alkyl groups of different sizes have been used as probes for assessing the steric accessibility of the heme iron in hemoproteins (St. George & Pauling, 1951; Ainsworth et al., 1960; Brunori et al., 1972; Stetzkowski et al., 1979; Reisberg & Olson, 1980a-c; Mims et al., 1983). Differences in the binding constants of alkyl isocyanides to model heme complexes (Olson et al., 1983) and hemoproteins have also indicated a partitioning effect of a hydrophobic heme environment on binding.

In the present study, we have measured the equilibrium constants for alkyl isocyanide binding to ferrous cytochrome *c'* from *Rs. molischianum* in order to determine the extent to which steric effects and hydrophobic residues indicated in the X-ray structure of the ferric cytochrome *c'* influence ligand binding in this protein relative to other hemoproteins and model heme complexes.

## Materials and Methods

*Rhodospirillum molischianum* cytochrome *c'* was prepared according to the procedure of Bartsch (1978). Methyl, ethyl, and propyl isocyanides were synthesized according to the procedure of Casanova and Schuster (1963). *n*-Butyl, *tert*-butyl, and hexyl isocyanides were purchased from Aldrich. Amyl isocyanide was purchased from Dixon Fine Chemicals. Amyl and hexyl isocyanides were further redistilled before use. Equilibrium measurements were carried out in a standard semimicro rectangular cuvette fitted with a Teflon stopper. One milligram of sodium dithionite was added to the cuvette containing 1.0 mL of the ferric cytochrome *c'* at $\sim 4 \times 10^{-6}$ M in 0.1 M potassium phosphate buffer at pH 7.0 to reduce and maintain the iron in the ferrous state. The reduced cytochrome *c'* solution was titrated with pure alkyl isocyanide by using a 25-$\mu$L Hamilton syringe with a repeating dispenser attachment. All spectral measurements were made in the visible region (650-350 nm) on a Cary 14 or Cary 17 spectrophotometer after equilibrium was achieved. Absorption changes measured at the Soret absorption maximum for the cytochrome *c'* complex were used to determine the equilibrium

† This work was supported by National Institutes of Health Grant GM 21277 (M.A.C.).

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0006-2960/89/0428-2140$01.50/0 

constant from the slope of a plot of $A$ vs $(A - A_0)/L$, consistent with the expressions:

$$\text{cyt } c' + \text{L} \rightleftharpoons \text{cyt } c'\text{-L}$$

$$K = \frac{[\text{cyt } c'\text{-L}]}{[\text{cyt } c'][\text{L}]}$$

$$\frac{[\text{cyt } c'\text{-L}]}{[\text{cyt } c']} = \frac{A - A_0}{A_{100} - A}$$

where $A_0$ and $A_{100}$ are the absorbances at 0% and 100% complex formation, respectively, and $A$ is the absorbance corresponding to an intermediate ligand concentration.

$$K = \frac{(A - A_0)}{(A_{100} - A)} \frac{1}{[\text{L}]}$$

$$A = \frac{(A_0 - A)}{[\text{L}]} \frac{1}{K} + A_{100}$$

Absorption maxima for each isocyanide complex were obtained from computer-generated values of $A_{100}$ from the $A$ of a partially complexed cytochrome solution measured at 0.2-nm intervals at wavelenths from 650 to 350 nm, the average equilibrium constant determined from several titrations, and ligand concentration of the solution according to the equation:

$$A_{100} = A - \frac{A_0 - A}{[\text{L}]K}$$

Extinction coefficients of the cytochrome $c'$ complexes were calculated from computed values of $A_{100}$ and the concentration of the protein based on reported extinction coefficients for the reduced, unligated protein.

Molecular weights were determined by molecular sieve chromatography using an LKB HPLC system equipped with a Tsk-Gel column, type G2000SW (7.5 mm × 500 mm). Horse heart myoglobin, sperm whale myoglobin, cytochrome $c$, trypsin, and bovine serum albumin obtained from Sigma were used as standards to obtain a plot of log MW vs elution time. The buffer for standards was 100 mM potassium phosphate at pH 7.0. The column was equilibrated with phosphate buffer containing sodium dithionite to obtain the elution volume of the unligated cytochrome $c'$ and, additionally, with $n$-butyl isocyanide to obtain the elution volume of ligated cytochrome $c'$.

## Results

Alkyl isocyanide binding to *Rs. molischianum* cytochrome $c'$ is associated with a shift in the Soret absorption maximum from 423 to 425–428 nm and a large increase in extinction coefficient in the Soret band. The range of observed absorption maxima and calculated extinction coefficients for the alkyl isocyanide complexes of *Rs. molischianum* cytochrome $c'$ as well as ethyl isocyanide complexes of other cytochromes $c'$ (Rubinow & Kassner, 1984) are presented in Table I. The visible extinction coefficients for the alkyl isocyanide complexes decrease from 17.1 (556 nm) and 20.2 (525 nm) for methyl isocyanide to 11.7 (556 nm) and 11.9 (525 nm) for hexyl isocyanide. The extinction coefficients for the Soret band of the alkyl isocyanide complexes of *Rs. molischianum* cytochrome $c'$ are similar to those observed for *Rh. palustris* and *Rs. rubrum* but much greater than those of *C. vinosum* and *Rh. sphaeroides*. Correspondingly, the half-bandwidths of the alkyl isocyanide complexes of *Rs. molischianum* are similar to those of the *Rh. palustris* and *Rs. rubrum* but much smaller than those of the *C. vinosum* and *Rs. sphaeroides* proteins (Rubinow & Kassner, 1984).

Figure 1 shows a typical spectrophotometric titration of reduced cytochrome $c'$ with ethyl isocyanide at pH 7.0 and 25 °C. Figure 2 illustrates a typical plot of $A$ vs $(A - A_0)/L$ and is consistent with the binding of one ligand according to the equilibrium expression

$$\text{cyt } c' + \text{L} \rightleftharpoons \text{cyt } c'\text{-L}$$

Table II shows the equilibrium constants for the binding of alkyl isocyanides and CO to *Rs. molischianum* cytochrome $c'$ compared to other heme proteins (Mims et al., 1983) and

Table I: Absorption Maxima and Extinction Coefficients of Alkyl Isocyanide Complexes of Cytochrome $c'$

| | absorption max (nm) (extinction coeff [$mM^{-1}$ heme $^{-1}$ $cm^{-1}$]) [half-bandwidth (nm)] | | |
|---|---|---|---|
| *Rs. molischianum* | | | |
| $c'$ | 549 | 432 (s) | 423 |
| $c'$-RIC[a] | 556 (13.4) | 525 (16.4) | 426 (282) [9.1] |
| *C. vinosum*[b] | | | |
| $c'$-EIC | 557 (18) | 527 (17) | 427.5 (189) [14.4] |
| *Rh. sphaeroides*[b] | | | |
| $c'$-EIC | 556 (21.4) | 527 (20.2) | 426.5 (169) [16.6] |
| *Rh. palustris*[b] | | | |
| $c'$-EIC | 555 (17) | 526.5 (17.9) | 427 (321) [7.6] |
| *Rs. rubrum*[b] | | | |
| $c'$-EIC | 554.5 (19.2) | 525.5 (19.5) | 426 (245) [10.6] |

[a] Absorption maxima and extinction coefficients (5–13% deviation) correspond to average values for all the alkyl isocyanides. [b] Rubinow & Kassner, 1984.

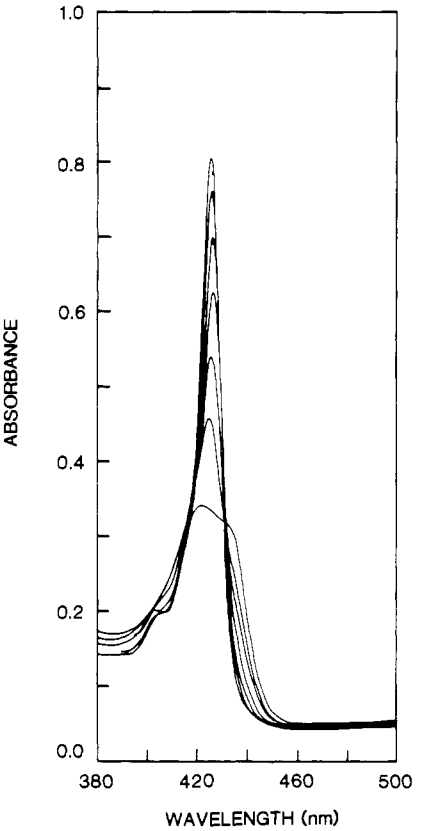


FIGURE 1: Spectrophotometric titration of ferrous cytochrome $c'$ from *Rs. molischianum* with ethyl isocyanide in 0.1 M potassium phosphate buffer at pH 7.0 and 25 °C.

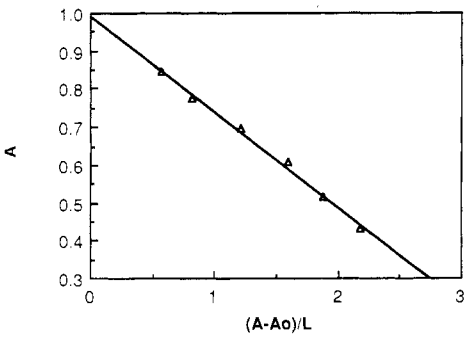


FIGURE 2: Plot of $A$ vs $(A - A_0)/L$ for the spectrophotometric titration of ferrous cytochrome $c'$ from *Rs. molischianum* with ethyl isocyanide in 0.1 M potassium phosphate buffer at pH 7.0 and 25 °C.

Table II: Comparison of Equilibrium Constants ($M^{-1}$) for Alkyl Isocyanide and CO Binding to *Rs. molischianum* Cytochrome $c'$ and Heme Complexes

| RIC | *Rs. molischianum* $c'$[a] | hemoglobin[b] | myoglobin[c] | model[c] |
|---|---|---|---|---|
| methyl | 8.7 | $3 \times 10^3$ | $2.3 \times 10^4$ | $0.11 \times 10^8$ |
| ethyl | 3.3 | $19 \times 10^3$ | $17 \times 10^4$ | $0.34 \times 10^8$ |
| propyl | 13 | $7.5 \times 10^3$ | $7.8 \times 10^4$ | $0.74 \times 10^8$ |
| butyl | 59 | $2.8 \times 10^3$ | $3.7 \times 10^4$ | $2.84 \times 10^8$ |
| amyl | 95 | $4 \times 10^3$ | $6.7 \times 10^4$ | $6.15 \times 10^8$ |
| hexyl | 260 | $8.9 \times 10^3$ | $16.6 \times 10^4$ | $15.1 \times 10^8$ |
| *tert*-butyl | 15 | $0.14 \times 10^3$ | $0.17 \times 10^4$ | $0.78 \times 10^8$ |
| cyclohexyl | 100 | $0.50 \times 10^3$ | $1.63 \times 10^4$ | $4.98 \times 10^8$ |
| CO | $4 \times 10^4$[d] | $2 \times 10^7$ | $3.3 \times 10^7$ | $3.90 \times 10^8$ |

[a] Deviation of 5–13%. [b] Reisburg & Olson, 1980. [c] Mims et al. (1983). Protoheme mono-3-(1-imidazoyl)propylamide monomethyl ester in 2% myristyltrimethylammonium bromide. [d] Doyle et al., 1985.

sterically unconstrained chelated protoheme model (Olson et al., 1983). The equilibrium constants for *Rs. molischianum* follow the order methyl > ethyl < *n*-propyl < *tert*-butyl < *n*-butyl < amyl < cyclohexyl < *n*-hexyl. The *Rs. molischianum* cytochrome $c'$ is the only protein that exhibits a greater binding constant for methyl isocyanide than ethyl isocyanide, 8.7 $M^{-1}$ and 3.3 $M^{-1}$, respectively. The ethyl isocyanide binding constant for *Rs. molischianum* $c'$ is $10^7$-fold less than the sterically unconstrained model, $\sim 10^3$-fold less than for hemoglobin, and $\sim 10^4$-fold less than for myoglobin. The ethyl isocyanide binding constant for the *Rs. molischianum* $c'$, 3.3 $M^{-1}$, is similar to the value of 2.2 $M^{-1}$ for *Rh. palustris* $c'$ and 1.2 $M^{-1}$ for *Rs. rubrum* $c'$ and $10^3$-fold less than binding constants for *C. vinosum* and *Rh. sphaeroides*, which have values of $3.3 \times 10^3$ $M^{-1}$ and $3.9 \times 10^3$ $M^{-1}$, respectively (Rubinow & Kassner, 1984).

The elution profiles for the unligated ferrous cytochrome $c'$ from *Rs. molischianum* and its *n*-butyl isocyanide complex were obtained on an HPLC molecular sieve column. The unligated and the ligated protein had elution times of 15.8 and 15.6 min, respectively, corresponding to molecular weights of 32 000 and 34 000.

## Discussion

Alkyl isocyanide complexes of *Rs. molischianum* cytochrome $c'$ have absorption spectra similar to those observed for ethyl isocyanide complexes of cytochrome $c'$ from *Rh. palustris* and *Rs. rubrum*. The absorption spectra of these complexes are characterized by narrow half-bandwidths and large extinction coefficients, compared to the spectra of ethyl isocyanide complexes of the cytochromes $c'$ from *C. vinosum*, *Rh. sphaeroides* (Rubinow & Kassner, 1984), and other heme proteins. In an earlier study of ethyl isocyanide binding to the cytochromes $c'$ from *Rh. palustris*, *Rs. rubrum*, *Rh. sphaeroides*, and *C. vinosum*, a correlation was observed between the equilibrium binding constants and the absorption spectra. The Soret half-bandwidths and the extinction coefficients for *C. vinosum* and *Rh. sphaeroides* exhibit more than a 2-fold difference compared to those of *Rh. palustris* and *Rs. rubrum*; correspondingly the binding constants for *C. vinosum* and *Rh. sphaeroides* are $10^3$-fold higher than for *Rh. palustris* and *Rs. rubrum* (Rubinow & Kassner, 1984). Additionally, the ratio of CO to ethyl isocyanide binding constants for cytochrome $c'$ from *Rs. sphaeroides* and *C. vinosum* are similar to a sterically unconstrained model heme complex and much smaller than the ratio of binding constants for *Rh. palustris* and *Rs. rubrum*, indicating that differences in electronic factors do not determine the difference in CO and ethyl isocyanide binding properties (Rubinow & Kassner, 1984). It was proposed that a sterically hindered heme ligand environment in *Rs. rubrum* and *Rh. palustris* contributed to the lower ethyl isocyanide binding affinity and greater Soret extinction coefficient with narrower half-bandwidth. As indicated in Table II, the ethyl isocyanide binding constant for *Rs. molischianum* (3.3 $M^{-1}$) is similar to that for *Rh. palustris* (2.2 $M^{-1}$) and *Rs. rubrum* (1.2 $M^{-1}$), consistent with the proposed correlation. In the present study, equilibrium constants for the binding of a series of eight alkyl isocyanides to cytochrome $c'$ from *Rs. molischianum* have been measured to determine the extent to which steric constraints indicated in the X-ray structure affect ligand binding.

As previously considered, a decrease in the binding constant would be expected as the size of the alkyl group increases for a sterically hindered coordination site (Antonini & Brunori, 1971). The results of this study suggest that steric interactions contribute to the decrease in equilibrium constant from methyl to ethyl isocyanide. It is noteworthy that *Rs. molischianum* cytochrome $c'$ is the only protein examined to date for which the equilibrium constant is greater for methyl isocyanide than for ethyl isocyanide. Furthermore, as previously observed for ethyl isocyanide binding to *Rh. pulstris* and *Rs. rubrum*, the spectroscopic properties of ethyl isocyanide binding to *Rs. molischianum* are consistent with a more restricted heme environment (Rubinow & Kassner, 1984). The increase in equilibrium constant from ethyl to hexyl may be associated with a hydrophobic heme coordination site in the protein, similar to that for the binding of alkyl isocyanides to a sterically unconstrained pentacoordinated protoheme model in aqueous detergent micelle (Olson et al., 1983). The differences in equilibrium constant for alkyl isocyanide binding to the model in detergent have been correlated with a difference in partition coefficients of the isocyanide between the micelle and the aqueous phase. The observed binding constants for the model increased as the size of the alkyl group increased. The present results suggest then that both steric and hydrophobic effects are operative in alkyl isocyanide binding to cytochrome $c'$ from *Rs. molischianum*. Similar conclusions were made for the binding of alkyl isocyanides to hemoglobin (Resiberg & Olson, 1980a).

Mims et al. (1983) evaluated the extent of steric interaction between the alkyl isocyanide and protein in hemeproteins by considering the differences between the free energies of ligand binding to a chelated protoheme model in a soap micelle and to the heme protein according to the expression:

$$\Delta G_{PROT} = RT \ln (K_{soap}/K_{obsd})$$

$\Delta G_{PROT}$ is a measure of both the steric interaction between the ligand and the protein at the sixth coordination site and the proximal effect at the fifth coordination site. This type of analysis is based on the assumption that the hydrophobic

Table III: Free Energy Parameters for Alkyl Isocyanide Binding to *Rs. molischianum* Cytochrome $c'$ and Heme Complexes

| | $\Delta G_{PROT}$ (kcal/mol) | | | |
|---|---|---|---|---|
| ligand | *Rs. molischianum* $c'$ | Mb[a] | Hb (T state)[a] | Hb (R state)[a] |
| CO | 5.35 | 1.43 | 3.47 | −0.30 |
| methyl | 8.2 | 3.59 | 5.94 | 2.41 |
| ethyl | 9.4 | 3.10 | 5.73 | 2.02 |
| *n*-propyl | 9.1 | 3.99 | 6.57 | 3.03 |
| *n*-butyl | 9.0 | 5.20 | 7.46 | 4.37 |
| amyl | 9.1 | 5.31 | 7.42 | 4.58 |
| hexyl | 9.1 | 5.31 | 7.16 | 4.66 |
| *tert*-butyl | 9.0 | 6.24 | 8.37 | 5.27 |
| cyclohexyl | 9.0 | 6.01 | | |

[a] Mims et al. (1983). Average of $\alpha$ and $\beta$ subunits.

environment in the micelle is similar to that of the protein and that the homologous series of alkyl isocyanides and CO will equally be affected by any proximal effect. Therefore, differences in $\Delta G_{PROT}$ between CO and alkyl isocyanides are a measure of steric effects between the alkyl isocyanides and the protein at the sixth coordination site. Table III shows the $\Delta G_{PROT}$ values for the binding of CO and alkyl isocyanides to hemoglobin, myoglobin, and *Rs. molischianum* cytochrome $c'$. In the absence of any steric effect, $\Delta G_{PROT}$ would be constant for each of the alkyl isocyanides. An increase in $\Delta G_{PROT}$ values as the size of the ligand increases is consistent with an increase in steric interaction as previously observed for hemoglobin and myoglobin (Mims et al., 1983).

The $\Delta G_{PROT}$ for CO binding to *Rs. molischianum* cytochrome $c'$ is 5.35 kcal/mol compared to 1.43 kcal/mol for myoglobin, ~0.0 kcal/mol for R-state hemoglobin, and 3.4 kcal/mol for T-state hemoglobin. The 3.4 kcal/mol increase in going from the R to T state and the 1.43 kcal/mol for myoglobin has been interpreted in terms of an unfavorable proximal effect on the basis of the uniform dependence of $\Delta G_{PROT}$ for these proteins on ligand size and shape and the rate constants for complex formation (Mims et al., 1983). The rate constants for CO binding to cytochromes $c'$ (Cusanovich & Gibson, 1973) are $10^4$–$10^6$-fold slower than that for the model heme complex and $10^3$–$10^4$-fold slower than those for hemoglobin and myoglobin, which suggests that the very large $\Delta G_{PROT}$ of 5.35 kcal/mol observed for CO binding to cytochrome $c'$ from *Rs. molischianum* is more likely due to a steric effect than to a proximal effect. The $\Delta G_{PROT}$ for the binding of methyl isocyanide to *Rs. molischianum* cytochrome $c'$ is ~3 kcal/mol greater than that for CO binding. Since electronic differences between model heme and heme protein would be expected to similarly affect CO and methyl isocyanide, the large increase in the value of $\Delta G_{PROT}$ from CO to methyl isocyanide for *Rs. molischianum* cytochrome $c'$ is due to further steric interaction caused by addition of the third ligand atom. The increase in $\Delta G_{PROT}$ from CO to methyl isocyanide for *Rs. molischianum* cytochrome $c'$ is somewhat greater than that observed for hemoglobin and myoglobin. Addition of the fourth ligand atom, from methyl to ethyl isocyanide, increases $\Delta G_{PROT}$ ~1.2 kcal/mol and is also consistent with a further increase in steric interaction from methyl to ethyl isocyanides, corresponding to a decrease in the affinity constant. Extending the alkyl chain from ethyl to hexyl or branching at the $\alpha$-carbon causes no additional changes in $\Delta G_{PROT}$, which suggests that no additional steric interactions are present.

The results indicate that the extremely small equilibrium constants observed for the binding of methyl and other alkyl isocyanides to *Rs. molischianum* ferrous cytochrome $c'$ are in large part associated with a heme coordination site that is severely sterically hindered. Figure 3 shows a schematic representation of the steric constraints to ligand binding at the sixth coordination site of *Rs. molischianum* ferrous cytochrome $c'$, hemoglobin, and myoglobin based on differences in free energies of binding relative to the sterically unrestricted model complex. A comparison of data for cytochrome $c'$ from *Rs. molischianum* to those for hemoglobin and myoglobin suggests that the binding site in cytochrome $c'$ is much less accessible. These observations are similar to the heme coordination site reported for the crystal structure of *Rs. molischianum* ferric cytochrome $c'$. Comparison of the ethyl isocyanide binding constant for *Rs. molischianum* cytochrome $c'$ to those of *Rh. sphaeroides* and *C. vinosum* cytochrome $c'$ suggests the distal heme binding site in these latter proteins is more accessible.

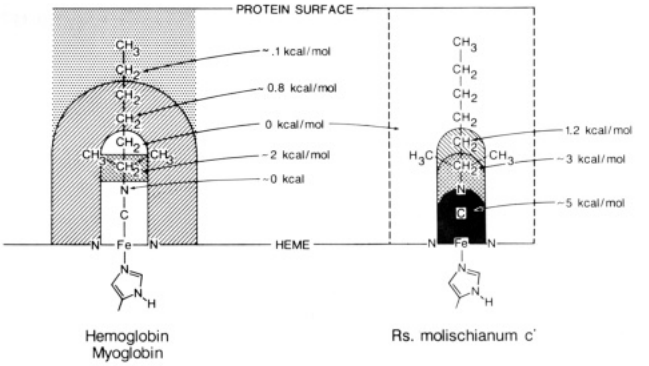


FIGURE 3: Schematic representation of the free energy differences associated with steric interactions at the sixth coordination site of *Rs. molischianum* cytochrome $c'$ based on alkyl isocyanide and CO binding compared to the site reported for hemoglobin and myoglobin by Mims et al. (1983).

**Registry No.** Cyt *c*, 9035-41-0; methyl isocyanide, 593-75-9; ethyl isocyanide, 624-79-3; propyl isocyanide, 627-36-1; butyl isocyanide, 2769-64-4; amyl isocyanide, 18971-59-0; hexyl isocyanide, 15586-23-9; *tert*-butyl isocyanide, 7188-38-7; cyclohexyl isocyanide, 931-53-3.

## References


Ainsworth, S., Gibson, Q. H., & Roughton, F.J.W. (1960) *Proc. R. Soc. London, B 152*, 331–345.

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.

Bartsch, R. G. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) Plenum Press, New York.

Brunori, M., Talbot, B., Colosimio, A., Antonini, E., & Wyman, J. (1972) *J. Mol. Biol. 65*, 423–434.

Casonova, J. J., Schuster, R. E., & Werner, N. D. (1963) *J. Chem. Soc.*, 4280–4281.

Casonova, J. J., Werner, R. E., & Schuster, R. E. (1966) *J. Org. Chem. 31*, 3473–3482.

Cusanovich, M. A., & Gibson, Q. H. (1973) *J. Biol. Chem. 248*, 822–834.

Doyle, M. L., Weber, P. C., & Gill, S. J. (1985) *Biochemistry 24*, 1987–1991.

Doyle, M. L., Gill, S. J., & Cusanovich, M. A. (1986) *Biochemistry 25*, 2509–2516.

Doyle, M. L., Gill, S. J., Meyer, T. E., & Cusanovich, M. A. (1987) *Biochemistry 26*, 8055–8058.

Finzel, B. C., Weber, P. C., Hardman, K. D., & Salemme, F. R. (1985) *J. Mol. Biol. 186*, 627–643.

Kassner, R. J., Rubinow, S. C., & Cusanovich, M. A. (1983) *Biochim. Biophys. Acta 743*, 195–199.

Mims, M. P., Porras, A. G., Olson, J. S., Noble, R. W., & Peterson, J. A. (1983) *J. Biol. Chem. 23*, 14219–14232.

Olson, J. S., Mims, M. P., & Reisberg, P. I. (1982) in *Hemoglobin and Oxygen Binding* (Ho, C., Ed.) pp 393–398, Elsevier/North-Holland, New York.
Olson, J. S., McKinnie, R.-E., Mims, M. P., & White, D. K. (1983) *J. Am. Chem. Soc. 105*, 1522–1527.
Reisberg, P. C., & Olson, J. S. (1980a) *J. Biol. Chem. 255*, 4144–4150.
Reisberg, P. C., & Olson, J. S. (1980b) *J. Biol. Chem. 255*, 4151–4158.
Reisberg, P. C., & Olson, J. S. (1980c) *J. Biol. Chem. 255*, 4159–4169.
Rubinow, S. C., & Kassner, R. J. (1984) *Biochemistry 23*, 2590–2595.
Stetzkowski, F., Cassoly, R., & Banerjee, R. (1979) *J. Biol. Chem. 254*, 11351–11356.
St. George, R. C. C., & Pauling, L. (1951) *Science 114*, 629–634.
Taniguchi, S., & Kamen, M. D. (1963) *Biochim. Biophys. Acta 74*, 438–445.
Weber, P. C., Bartsch, R. G., Cusanovich, M. A., Hamlin, R. C., Howard, A., Jordon, S. R., Kamen, M. D., Meyer, T. E., Wertherford, D. U., Xuong, N. H., & Salemme, F. R. (1980) *Nature (London) 286*, 302–304.
Weber, P. C., Howard, A., Xuong, N. H., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399–424.

# Hysteresis and Conformational Drift of Pressure-Dissociated Glyceraldehydephosphate Dehydrogenase†

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ABSTRACT: Pressure dissociation of yeast glyceraldehydephosphate dehydrogenase (GAPDH) was studied by fluorescence spectroscopy. Observations in the range of −5 to 30 °C indicate that monomer association into the tetramer proceeds with an enthalpy change of −14 kcal mol$^{-1}$ and a large increase in entropy which at 25 °C amounts to 18 kcal mol$^{-1}$. The large conformational drift and the low-temperature stability of the tetramer recovered after decompression facilitated a comparison of its properties with those of the native tetramer. Significant differences in absorption and fluorescence-excitation polarization spectra, yield of tryptophan fluorescence, and binding of anilinonaphthalenesulfonate and NADH were observed. At 0 °C the standard free energies of association of the monomers into the native and drifted tetramers were respectively −32 and −29 kcal mol$^{-1}$. The volume change upon association measured from the pressure span of the compression curves was 200–230 mL mol$^{-1}$ but four times as large when derived from the displacement of the compression curves with total protein concentration. This large discrepancy can be explained by the existence in the native tetramer population of a distribution of free energies of association with a dispersion from the mean of about 6 kcal mol$^{-1}$. At 0 °C and 1 bar ATP and ADP decreased the stability of the GAPDH tetramer by changes in free energy of association of +3.7 and +4.1 kcal mol$^{-1}$, respectively. NAD and c-AMP stabilized it by −2.3 and −1.3 kcal mol$^{-1}$. The variation in sign and magnitude of the ligand-induced changes in free energy of association observed in this case, and previously in hexokinase [Ruan, K., & Weber, G. (1988) *Biochemistry 27*, 3295], and the heterogeneity of the free energy of association of GAPDH, revealed as indicated above, lead to the conclusion that oligomeric aggregates exist in a variety of conformations that depend upon the protein concentration, temperature, pressure, and the presence of specific ligands. The multiplicity of species revealed by the energetics raises questions about the significance of the structures of oligomeric proteins determined by X-ray crystallography.

## Theory

The thermodynamic dissociation constant $K$ of the reaction involving an equilibrium between tetramer and monomers is

$$K = 256a^4C^3/(1 - a) \quad (1)$$

where $a$ is the degree of dissociation into monomers and $C$ the protein concentration, as tetramer. For practical purposes $K$ may be conveniently expressed as a protein concentration $C_{1/2}$ at which $a = 1/2$:

$$C_{1/2}{}^3 = (K/32) = 8a^4C^3/(1 - a) \quad (2)$$

The free energy[1] of monomer *association* into the tetramer is

$$\&G = RT \ln (32C_{1/2}{}^3) = RT(3.467 + 3 \ln C_{1/2}) \quad (3)$$

If $C_{1/2}$ and $a_P$, respectively, denote the half-dissociation concentration at atmospheric pressure and the degree of dissociation at pressure $p$

$$\ln (a_P{}^4/(1 - a_P)) = \ln (C_{1/2}/2C)^3 + p\&V/RT \quad (4)$$

and a plot of the left-hand side of eq 4 against the applied

† This research was supported by a grant from the National Institutes of Health (GM 11223) to G.W.

[1] Abbreviations: GAPDH, glyceraldehydephosphate dehydrogenase; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; c-AMP, cyclic adenosine 5′-monophosphate; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)-aminomethane; ANS, 1-anilino-8-naphthalenesulfonate; 2,5-DNS, 2-(dimethylamino)-5-naphthalenesulfonyl chloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol. To lighten the notation and to avoid confusing superscripts, we refer to the standard molar changes in free energy, enthalpy, entropy, and volume, respectively, by $\&G$, $\&H$, $\&S$, and $\&V$.

0006-2960/89/0428-2144$01.50/0 