Criteria for Evaluating Enzymic Rate Enhancements. The Case of Glyceraldehyde-3-phosphate Dehydrogenase

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Abstract: The evaluation of a rate enhancement for the reaction of an irreversible inhibitor or a substrate with the enzyme relative to the reaction with a model compound depends on the choice of the model compound. The model compound should not deviate in reactivity from similar model compounds. This can be determined from the appropriate linear-free-energy relationship. The rate enhancement is the ratio of the enzymic rate constant to the rate constant for the reaction of the model compound *with the same* pK_a as the residue on the enzyme. The effect of substituents on the inhibitor or substrate on this rate enhancement resulting from specific interactions of the substrate with the protein can be evaluated from a Hammett-type linear-free-energy relationship obtained with a variety of substituted reactants. This corrects for any differences in the sensitivities of the enzymic and nonenzymic reactions to the electronic effects of the substituents. A phosphate substituent on the substrate, or on irreversible inhibitors, of glyceraldehyde-3-phosphate dehydrogenase enhances the reactivity of the protein. This enhanced reactivity, corresponding to a lowering of the activation energy barrier by 3.8 (±0.1) kcal/mol, results from a specific interaction of the phosphate moiety of the reactant with the enzyme.

Enzymes are very efficient catalysts. Analysis of the factors responsible for this high catalytic efficiency of enzymes has led to a better understanding of the mechanism of action of enzymes and, indeed, of chemical reactions in general. Most enzymes contain at least one essential amino acid residue at the active site which is unusually reactive.[1] An estimation of the enhanced reactivity of a particular amino acid residue at the active site of the enzyme which may serve as a nucleophile in the catalytic reaction is often made by comparing the rate of reaction of an alkylating reagent with the residue in the enzyme and with a model compound. However, such comparisons can often be misleading if certain factors (such as the appropriateness of the model compound and the relative sensitivities of the enzymic and model reactions to substituent effects) are not taken into consideration. A careful analysis of these factors allows the evaluation of the individual components of the reaction which contribute to the catalytic efficiency of the enzymic reaction.

Experimental Section

Yeast glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12.) was prepared as described previously.[2] The rates of reaction of the alkylating reagents with *p*-nitrothiophenol were followed spectroscopically by the decrease in absorbance at 412 nm. The second-order rate constants for the reaction of the other model thiols with the alkylating reagents were determined spectrophotometrically by titration of the unreacted thiol with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[3] For the reaction of the thiols with iodoacetamide the reaction mixture contained 0.05 M buffer [ethylenediamine, *N,N′*-bis(2-hydroxyethyl)glycine, or triethylamine], 0.1 M KCl, 1–10 mM iodoacetamide, and 1–2 mM thiol. The mixture was incubated at pH 6–11 and 25.0 °C (±0.1 °C) and aliquots were removed at various times and diluted 20-fold into a cuvette containing 0.1 M sodium pyrophosphate (pH 8.5) and 4×10^{-4} M DTNB. Absorbance at 412 nm was monitored in a Beckman DU spectrophotometer modified with an Update Model 122 digital display log converter amplifier. The reactions with the less reactive alkylating reagents were followed under pseudo-first-order conditions. The rates of reaction of the alkylating reagents with the enzyme were obtained by following the loss of enzymic activity.

The diethyl acetal of glycidaldehyde was prepared by the method of Williams et al.,[4] bp 43–45 °C (4.5 mm) [lit. bp 115–117 °C (140 mm)]. Chloroacetol phosphate (prepared by the method of Hartman[5]) was a generous gift from Dr. Hartman. Other reagents were obtained from Aldrich Chemical Co. or prepared as described earlier.[6]

Results and Discussion

I. Choice of the Model Compound. The analysis and interpretation of enzymic rate enhancements are dependent on controlled chemical studies for comparison. Particularly crucial is the choice of the model compound.

Clearly, the model compound should have the same reactive residue as the moiety at the active site of the enzyme. Thus, for an enzyme with a sulfhydryl group at the active site the model compound should have a sulfhydryl group. Glutathione and *N*-acetylcysteine are often used as model thiols for such a comparison. However, several factors concerning the appropriateness of the model thiol must be considered. Some of these are quite obvious; the products of the enzymic and model reactions must be identical and the concentration of the reactive species must be known (e.g., if the reactive species is the thiolate anion the pK_a's of the enzyme and the model compound must be evaluated under the same experimental conditions). However, the pK_a of the thiol will affect not only the concentration of the thiolate present but also the nucleophilic reactivity of the thiolate, depending on the Brønsted coefficient, β, for the reaction.

For example, in an interesting study of the reactivity of the thiol residue at the active site of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with iodoacetamide, MacQuarrie and Bernhard[7] analyzed the pH profile for the alkylation of the enzyme and the alkylation of *N*-acetylcysteine. The reactive species is the thiolate anion. Thus, the observed rate constant for the reaction at any given pH is given by the expression

$$k_{obsd} = k^{lim}/(1 + [H^+]/K_a) \quad (1)$$

where k^{lim} is the limiting second-order rate constant for the reaction of iodoacetamide with a thiolate anion with a dissociation constant of K_a. Both the reaction with the enzyme and with *N*-acetylcysteine follow second-order kinetics (i.e., iodoacetamide does not bind to the enzyme prior to alkylation). The limiting rate constant for the reaction with the enzyme is 178 M^{-1} s^{-1} and with the model thiol the rate constant is 33 M^{-1} s^{-1}. Thus, it might be concluded that the enzyme SH group is 5.4 times more reactive than the *N*-acetylcysteine SH group. In fact, this figure is an underestimate for the enzymic rate enhancement.

When a plot of the logarithm of the second-order rate constant (obtained under the same conditions employed by MacQuarrie and Bernhard, viz., 0.1 M KCl, 25 °C) for the reaction of a series of thiolate anions (*p*-nitrothiolphenol, thiophenol, *N,N*-dimethylcystamine, glutathione, *N*-acetylcysteine, and β-mercaptoethanol) vs. the pK_a of the thiol is constructed, a straight line is obtained with a slope of 0.32.

Since *N*-acetylcysteine does not deviate from this linear-free-energy relationship it is an appropriate model thiol (i.e., the only factors responsible for the difference in rate constants for the reaction with *N*-acetylcysteine and with the other thiols are the same factors responsible for the difference in dissociation constants for the various thiols). However, since *N*-acetylcysteine ($pK_a = 9.52$) is more basic than the enzymic sulfhydryl group ($pK_a = 8.0$), and the β value for the reaction of the thiols with iodoacetamide is 0.32, this particular model thiol is expected to be about 3.1 times more reactive than a model thiol of $pK_a = 8.0$.

$$\log k^{lim} = \beta(pK_a) + c \quad (2)$$

$$\log k^{lim}\,(pK_a = 8.0) - \log k^{lim}\,(pK_a = 9.52) = \beta(8.0\text{–}9.52) \quad (3)$$

$$\log k^{lim}\,(pK_a = 8.0) = 0.32(-1.52) + \log\,(33) = 1.03 \quad (4)$$

$$\therefore k^{lim}\,(pK_a = 8.0) = 10.7\ M^{-1}\,s^{-1} \quad (5)$$

Thus, the actual rate enhancement for the reaction of the enzymic SH group with iodoacetamide, relative to that of a model thiol with the same pK_a as the enzyme, is 178/10.7 = 16.6. For the reaction of the yeast glyceraldehyde-3-phosphate dehydrogenase with iodoacetamide, the observed rate enhancement relative to *N*-acetylcysteine (calculated from the data of Byers and Koshland[8]) is 19.4. However, the pK_a of the active-site sulfhydryl residue in the yeast enzyme is 8.5[8] and, thus, the true rate enhancement is 41.4. This indicates that the active-site sulfhydryl group in the yeast enzyme is 2.5 (=41.4/16.6) times more reactive toward iodoacetamide than is the active-site sulfhydryl group of the rabbit muscle enzyme. Furthermore, this enhanced reactivity of the yeast enzyme relative to the rabbit muscle enzyme is due to factors other than differences in the pK_a of the active-site sulfhydryl residues.

This analysis depends on an accurate determination of the pK_a of the enzymic active-site residue. The evaluation of this pK_a is not always straightforward. The pH dependence of the kinetic parameter k_{cat}/K_m generally provides a correct evaluation of the pK_a value(s) of the active-site residue(s) in the free enzyme.[9,10] However, there are exceptions to this. For example, if there is a change in rate-limiting step to association of the enzyme and substrate with changing pH, there will be an anomalous dependence of $k_{cat.}/K_m$ on pH.[11] Furthermore, if the substrate can ionize, $k_{cat.}$ and/or K_m may be characterized by a pK_a unrelated to any residue on the enzyme (for example, see ref 12). This is also true for the pH profile for chemical modification of a particular active-site residue by an ionizable modifying reagent.[12] The pK_a of a particular active-site residue can, in principle, be determined unambiguously by direct spectroscopic techniques. The pH profile for the chemical modification of a particular amino acid residue with a series of nonionizable modifying reagents can also provide an evaluation of the pK_a of that residue. Any anomalous pK_a with a particular reagent is indicative of other factors, unique to that reagent, which perturb the pK_a of the amino acid residue (e.g., electrostatic interactions and/or conformational changes induced by a substituent on the reagent).

In some situations an active-site residue may have an inherently anomalous pK_a resulting from its local environment. This is one factor which may contribute to an enhanced reactivity of that residue. Analysis of the rate enhancement based on this pK_a value can lead to evaluation of factors other than the perturbation of the pK_a which may contribute to the overall rate enhancement.

II. Substituent Effects. When a series of structurally related irreversible inhibitors are available the inhibitor which is most structurally related to the substrate generally reacts fastest. Again, this rate enhancement is often underestimated. This will result if the enzymic reaction has a different sensitivity to the substituent than the nonenzymic reaction. This may be due to steric effects with the protein (e.g., steric exclusion of the active-site region or the presence of a "recognition" site on the enzyme) and to electronic effects (e.g., sensitivity to the microsolvent of the active-site region).

If the second-order rate constant for the reaction of an irreversible inhibitor, without any added substituents, is k_e^0 for the enzymic reaction and k_n^0 for the reaction with an appropriate model compound, then the presence of a substituent, x, will alter the rates of the reactions. If this effect is purely an electronic one (e.g., inductive) then application of the Hammett–Taft relationship (e.g., see Hammett[13]) yields

$$\log\,(k_e^x/k_e^0) = \rho_e^*\sigma_x^* \quad (6)$$

$$\log\,(k_n^x/k_n^0) = \rho_n^*\sigma_x^* \quad (7)$$

where σ_x^* is the Taft substituent constant for x and ρ_e^* and ρ_n^* are the sensitivities of the enzymic and nonenzymic reactions to the substituents. The parameter λ is defined as the ratio of these ρ^* values.

$$\frac{\log\,(k_e^x/k_e^0)}{\log\,k_n^x/k_n^0)} = \rho_e^*/\rho_n^* = \lambda \quad (8)$$

If the enzyme is insensitive to substituents (e.g., the rate-limiting step is diffusion together of the inhibitor and the enzyme), then $\lambda = 0$. The value of λ can be evaluated from a plot of log k_e^x vs. log k_n^x.

In general, the rate enhancement for reaction of an inhibitor with substituent x with the enzyme relative to the reaction with a model compound is given by rearranging eq 8.

$$k_e^x/k_n^x = k_e^0(k_n^x)^{\lambda-1}/(k_n^0)^{\lambda} \quad (9)$$

The differential electronic effects can now be separated from the other catalytic effects by application of transition state theory.[14] Thus

$$\log k_i = \log\,(h/kT) - \Delta G_i^{\ddagger}/2.3RT \quad (10)$$

where h is Planck's constant and k is Boltzmann's constant.

Equation 9, in logarithmic form, is

$$\log k_e^x - \log k_n^x = \log k_e^0 + (\lambda - 1)\log k_n^x - \lambda \log k_n^0 \quad (11)$$

Substitution of eq 10 into eq 11 yields

$$\Delta G^{\ddagger}_{n,x} - \Delta G^{\ddagger}_{e,x} = \Delta G^{\ddagger}_{e,0} - \lambda\Delta G^{\ddagger}_{n,0} + (\lambda - 1)\,\Delta G^{\ddagger}_{n,x} \quad (12)$$

where the subscripts for the free energy of activation ($\Delta G^{\ddagger}$) refer to the enzymic (e) or nonenzymic (n) reaction and whether the substituent is present (x) or absent (0).

From eq 7

$$\log k_n^x = \rho_n^*\sigma_x^* + \log k_n^0 \quad (13)$$

and from eq 10

$$\log k_n^x = \log\,(h/kT) - \Delta G^{\ddagger}_{n,x}/2.3RT \quad (14)$$

Equating eq 13 and 14 yields

$$\rho_n^*\sigma_x^* + \log k_n^0 = \log\,(h/kT) - \Delta G^{\ddagger}_{n,x}/2.3RT \quad (15)$$

But

$$\log k_n^0 = \log\,(h/kT) - \Delta G^{\ddagger}_{n,0}/2.3RT \quad (16)$$

Thus

$$\Delta G^{\ddagger}_{n,x} = -2.3RT\rho_n^*\sigma_x^* + \Delta G^{\ddagger}_{n,0} \quad (17)$$

Substituting eq 17 into eq 12 yields

$$\Delta\Delta G^{\ddagger}_x \equiv \Delta G^{\ddagger}_{n,x} - \Delta G^{\ddagger}_{e,x} = \Delta G^{\ddagger}_{e,0} - \Delta G^{\ddagger}_{n,0} - 2.3RT(\rho_e^* - \rho_n^*)\sigma_x^* \quad (18)$$

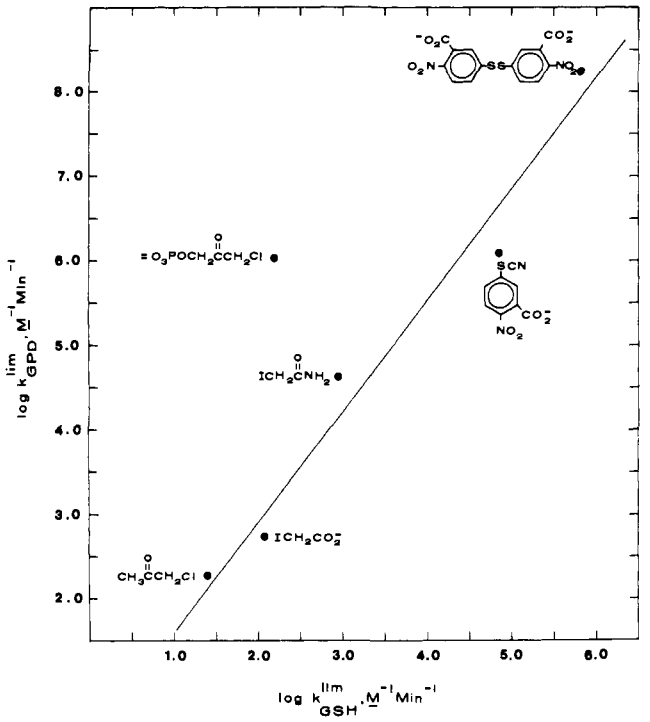
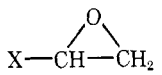


Figure 1. Substituent effects on modification of yeast glyceraldehyde-3-phosphate dehydrogenase. k_{GPD}^{lim} is the limiting second-order rate constant for reaction with the enzyme and k_{GSH}^{lim} is the limiting second-order rate constant for the reaction with glutathione. The reactions were carried out at 25 °C (±0.1°) and μ = 0.1 M. The line is for the equation log k_{GPD}^{lim} = λ log k_{GSH}^{lim} + C. The value of λ, obtained by a least-squares fit of the data excluding the point for chloroacetol phosphate, is 1.31 (r = 0.98). The interval estimator[16] of the slope (at 90% confidence) is ±0.291 (±22%). The standard deviations of the rate constants, based on at least three determinations, are ±3–7%. If the point for chloroacetol phosphate is included in the least-squares regression analysis, the interval estimator of the slope (λ = 1.11, r = 0.85) at 90% confidence is ±0.823 (±74%).

The term $\Delta G^{\ddagger}_{e,0} - \Delta G^{\ddagger}_{n,0}$ is a measure of the factors (other than differences in sensitivity to electronic factors) responsible for the differences in rates between the enzymic on nonenzymic reactions (e.g., steric factors, microsolvent effect, catalytic participation of other amino acid functional groups, cratic entropy).

The magnitude of the rate enhancement will depend on the choice of the unsubstituted reactant. If $\Delta G^{\ddagger}_{e,0} - \Delta G^{\ddagger}_{n,0} > 0$, then for some reactants a large rate enhancement may be observed but for others a rate decrease with the enzyme relative to the model compound may be observed. For example, the active-site SH group of yeast glyceraldehyde-3-phosphate dehydrogenase reacts with a variety of epoxides.[6] The presence of substituents on the epoxide



affects the rate of the reaction. None of the epoxides tested show saturation kinetics.

Glutathione is an appropriate model thiol for the reaction with the epoxides since the pK_a of glutathione (8.4) is within 0.1 unit of the pK_a of the yeast enzyme.[8] Under the experimental conditions of μ = 0.8 M, glutathione indeed falls on the Brønsted line (also see ref 15).

The ratios of k_e/k_n for various substituents on the epoxides are 0.16 [X = $-CH(OEt)_2$], 0.17 (X = $-CH_3$), 0.23 (X = $-CH_2OH$), 0.51 (X = $-CH_2Cl$), 0.72 (X = $-CHO$), and 1.1 (X = $-CH_2F$). At first glance, one is tempted to conclude that the enzyme can "recognize" a fluoro group more efficiently than a hydroxyl group or a hydrogen. However, a plot of log k_e vs. log k_n for these epoxides yields a straight line with a slope of λ = 1.74 (±15%, which is the interval estimator[16] at 90% confidence). This indicates that the only effect of the substituents on the rate enhancement is an electronic one with the enzymic reaction being more sensitive to the electronic effects than is the glutathione reaction. This may be a "slovent" effect since a decrease in solvent polarity will increase the ρ^* value for alkylation of thiols. Indeed, as indicated in Table I, 2-propanol (dielectric constant = 18.3) increases the ρ^* value for the reaction of p-nitrothiolphenol with epoxides. The 74% increase in ρ^* for the reaction of epoxides with the enzyme relative to model compounds is expected if the active-site environment of the enzymic sulfhydryl group is in a "microslovent" similar to ~7–9% (v/v) aqueous 2-propanol. This interpretation, however, cannot be a rigorous one since factors other than solvent effects (e.g., a different transition state structure for the enzymic and nonenzymic reactions) will contribute to a deviation of λ from unity. Indeed, Charney and Bernhard[18] have pointed out that spectral changes observed when a probe is placed at the active site of an enzyme can be due to factors other than differences in "solvent polarity".

Clearly the smaller k_e/k_n value with glycidol (0.23) than with epifluorohydrin (1.1) cannot be attributed to a steric effect since both epoxides fall on the same line (log k_e vs. log k_n). Indeed, only deviations from a plot of log k_e vs. log k_n are indicative of factors, other than electronic ones, which contribute to the rate constant of the enzymic reaction. The normal substrate for glyceraldehyde-3-phosphate dehydrogenase is the three-carbon aldehyde D-glyceraldehyde 3-phosphate. Placement of a phosphate moiety on the epoxide results in a rapidly reacting inhibitor, glycidol phosphate.[6] Glycidol phosphate is 20 times more reactive with the enzyme than with glutathione. However, because of the nature of the reaction ($\Delta G^{\ddagger}_{e,0} - \Delta G^{\ddagger}_{n,0} > 0$) and the differences in sensitivity of the enzymic and nonenzymic reaction to electronic effects (λ = 1.74) the actual rate enhancement for the reaction of glycidol phosphate with the enzyme (obtained from a plot of log k_e vs. log k_n) is ~630. Since the enzyme is "designed" to recognize a phosphate substituent (aldehydes containing a phosphate residue are over 600 times more reactive than the corresponding aldehydes lacking a phosphate residue[19]), this large positive deviation is not surprising.

Figure 1 illustrates the same effect of placing a phosphate residue on some nonepoxide inhibitors of the enzyme. In this case the λ value for the nonphosphorylated irreversible inhibitors is 1.31 (r = 0.98). Chloroacetol phosphate, however, shows a positive deviation corresponding to a rate enhancement

Table I. Solvent Effects on Reaction of p-Nitrothiophenol with Epoxides[a]

Solvent addition	k_0,[b] min^{-1} ± SEM	ρ^*, rel[c] (%)
None	1.50 ± 0.05	1.00
0.9 M NaCl	1.50 ± 0.04	0.96 (±28)
0.9 M sodium acetate	1.47 ± 0.05	1.07 (±30)
0.9 M LiCl	1.46 ± 0.05	1.04 (±23)
5% (v/v) 2-propanol	1.55 ± 0.03	1.50 (±18)
10% (v/v) 2-propanol	1.60 ± 0.05	1.95 (±20)
20% (v/v) 2-propanol	1.73 ± 0.06	~3.8 (±45)

[a] The reaction of various epoxides (propylene oxide, glycidol, epichlorohydrin, epifluorohydrin, glycidaldehyde, and glycidaldehyde diethyl acetal) with the thiol were carried out at pH 8.5 [0.05 M N,N'-bis(2-hydroxyethyl)glycine], 25.0 ± 0.1 °C. The pK_a of the thiol in the solvent without any additions is 5.1. [b] Second-order rate constant for the reaction of p-nitrothiophenol with propylene oxide based on three to five determinations. [c] Slope of a plot of log k(solvent + addition) vs. log k(solvent with no addition). The numbers in parentheses are the interval estimators[16] of ρ^* at 90% confidence.

of 679-fold. If the electronic effects are not taken into consideration the apparent rate enhancement, i.e., $(k_e/k_n)_{obsd}$, is actually larger (6250). The corrected rate enhancements obtained by placing a phosphate moiety on either the epoxide inhibitors or the inhibitors in Figure 1 correspond to a $\Delta\Delta G^{\ddagger}$ value ($\Delta G^{\ddagger}_n - \Delta G^{\ddagger}_e$) of 3.8 (±0.1) kcal/mol. This is a measure of the specific interaction of the phosphate moiety with the enzyme in the transition state.

Conclusion

The interpretation of an enzymic rate enhancement is dependent on the choice of the model compound. The model compound (a) should have the same reactive residue as present in the enzyme, (b) should produce the same product as that formed by reaction with the enzyme, (c) should not deviate in reactivity relative to other similar model compounds except with respect to electronic effects, and (d) should be normalized to the same electronic effects present in the enzymic residue (i.e., have a pK_a similar to that of the enzymic residue). The substituent effects on the reaction of the inhibitor (or the substrate) with the enzyme which contribute to this rate enhancement can be separated into two general factors: "electronic" and "steric". A plot of the logarithm of the enzymic rate constants versus the logarithm of the nonenzymic rate constants for a variety of substituted inhibitors (or substrates) should yield a straight line (if there is no change in the rate-limiting step with changing substituents). The slope, λ, is an index of the difference in electronic sensitivities of the enzymic and nonenzymic reactions and any deviations from this line indicate factors other than electronic ones which contribute to this rate enhancement.

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Communications to the Editor

Paramagnetic Anisotropy and Zero-Field Splitting in Tetraphenylporphyrinatoiron(III) Chloride

Sir:

Tetraphenylporphyrinatoiron(III) chloride (TPPFeCl) is a synthetic analogue of naturally occurring chlorohemin and resembles it very closely at least as far as the magnetic properties and coordination around the iron atom are concerned. In TPPFeCl the ferric ion is in high spin state ($S = 5/2$) with $^6S_{5/2}$ electronic ground state. The degeneracy of the sextet is partly removed by the combined effect of spin-orbit coupling and axial ligand field, to give three Kramers doublets, namely $M_s = \pm 1/2, \pm 3/2$, and $\pm 5/2$. A spin Hamiltonian of the form

$$\mathcal{H} = DS_z^2 \qquad (1)$$

gives the energy separation between the Kramers doublets $M_s = \pm 1/2$ and $M_s = \pm 3/2$ as $2D$, and that between $M_s = \pm 3/2$ and $M_s = \pm 5/2$ as $4D$, where D is the zero-field splitting (ZFS) parameter.

The ZFS is an important physical parameter in the high spin d^5 system. Its accurate determination has, therefore, evoked a continuing interest for a long time.[1] Several efforts have, for example, been made to determine accurately the ZFS in TPPFeCl and other hemin compounds. The average magnetic moment of TPPFeCl down to 2.2 K has been analyzed[2] on the basis of the above spin Hamiltonian, which gives $D = 11.8$ cm^{-1}. A similar value of D was obtained from the analysis of temperature dependence of isotropic proton shift studies on this compound.[3] The ZFS of a number of ferric porphyrins has also been determined directly by far-infrared techniques.[4] Though such measurements have not been reported for TPPFeCl, the ZFS in chlorohemin and protoporphyrin dimethyl ester iron(III) chloride has been determined very accurately by this technique, giving $D = 6.95$ cm^{-1} in both the compounds.[5] This value is evidently much smaller than the above value for the analogous TPPFeCl. The difference is especially surprising in the case of chlorohemin as its magnetic properties (down to 2.2 K) are very similar to TPPFeCl.[2] In view of this discrepancy and the interest in the ZFS in TPPFeCl, we have determined the ZFS in TPPFeCl from the measurements of paramagnetic anisotropy in the 80–300 K temperature range. Our measurements, contrary to the previous results, give $D = 5.9 \pm 0.1$ cm^{-1} in this compound.

TPPFeCl was prepared by the method described in the literature.[6] It was purified by column chromatography using a neutral silica-gel column (100–200 mesh) and using benzene solvent. The TPPFeCl gets adsorbed at the top of the column. It was eluted with benzene containing 10% (V/V) ethanol. The compound was characterized by elemental analysis and by its spectrum in the UV–visible region. The spectrum was taken in chloroform and was recorded on a Carl-Zeiss spectrophotometer. The peak positions agreed very well with those re-