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A Self-Validation Technique for Protein Structure Refinement: the Extended Hamilton Test

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Abstract

An extension is proposed for the self-validation Hamilton test [Hamilton (1965). *Acta Cryst.* **18**, 502–510] for crystallographic refinement. The method is based on the statistical **F** test and evaluates the significance of the *R*-factor ratio between two refinement protocols. The general case of two refinements carried out with different numbers and types of non-linear restraints is examined. The restraints are considered as extra observations weighted by a coefficient expressing their effective number. There exists a restriction on the weighting coefficients between the two refinements. An empirical method to evaluate the effective number of restraints is provided. The method may allow the detection of unreasonably tight restraints. The expectation value for r.m.s. R_{free}, given the r.m.s. R, can be estimated. Thus, the significance of the observed drop in R_{free} can be assessed. Compared to cross-validation using R_{free} [Brünger (1992). *Nature (London)*, **355**, 472–474] self-validation has the advantage that it does not require omission of any experimental data. The significance of the improvement obtained by moving from isotropic to anisotropic description of thermal parameters in the refinement of a protein at 1.5 Å resolution is used as an example.

1. Introduction

1.1. *Observations and parameters*

The refinement of a crystal structure is the minimization of the difference between the experimental electron-density distribution, obtained by Fourier transformation of structure factors derived from observed amplitudes and calculated phases, and a model represented by a set of variable parameters, which are refined. The refinement is typically performed in reciprocal space by least-squares minimization of the differences between observed and calculated structure factors. In refinement any increase in the number of independent parameters is reflected in better agreement between model and data, at the expense of a loss in the number of ways in which experimental errors can be accounted for, *i.e.* the number of degrees of freedom of the refinement defined as the number of observations minus the number of parameters refined. Since the number of parameters which optimally describe the experimental data is not known *a priori*, a validation procedure is required to avoid overfitting of the data.

This problem is particularly severe for proteins, where the number of observations available from the diffraction experiment is low with respect to the number of parameters typically used for the description of the structure. At a resolution lower than 2.5 Å the number of observations no longer exceeds the number of parameters. To overcome the problem of low data/parameters ratio, it is obligatory to increase the number of degrees of freedom of the refinement, either by augmenting the number of observations or by decreasing the number of parameters describing the model.

The former condition implies introduction of additional observational equations containing *a priori* information about the model in the form of restraints. Typically these are expressed as geometric or energetic conditions which the crystallographer might think the structure should satisfy (Waser, 1974; Sussman, Holbrook, Church & Kim, 1977; Konnert & Hendrickson, 1980). Restraints are represented by extra equations in the design matrix of the experiment and considered as observed data. Examples are: restraining bond distances to approach target values derived from the examination of accurately determined structures, *e.g.* the Cambridge Structural Database (Allen, Kennard & Taylor, 1983); restraining groups to be approximately planar (Urzhumtsev, 1991); restraining bonded atoms to have similar anisotropic thermal motion along the bond direction (Hirshfeld, 1976) or imposing non-crystallographic symmetry between chemically identical fragments (Bricogne, 1974). During refinement, as for X-ray data, restraints are given a weight which represents the distribution of parameters anticipated for their values.

The second way to increase the number of degrees of freedom of the refinement is to diminish the number of refined parameters. This is achieved by constraining them to have particular values or to obey exact conditions (Prince, Finger & Konnert, 1995). Examples are: imposing the space-group crystallographic symmetry; introducing H atoms at their calculated positions, riding on their carrier atoms; constraining occupancies of related complementary disordered atoms to sum up to one or constraining isotropic thermal parameters to

be equal within a group of atoms. Isotropic refinement can be considered as constrained anisotropic refinement, with the ellipsoids describing the atomic thermal motion constrained to be spheres, reducing the number of atomic thermal displacement parameters from six to one.

Thus, the number of degrees of freedom of the refinement can be tuned by varying the number of restraints and constraints. It is necessary to ensure that this number is the best choice to describe the data, by carrying out a validation procedure in parallel with the refinement.

1.2. *Cross- and self-validation*

Two general kinds of validation methods are self- and cross-validation. In self-validation descriptors are defined, either in reciprocal or in real space, to assess the quality of the refinement procedure. Examples are the R factor, 'a sort of average relative discrepancy between the observed and calculated values' (Hamilton, 1964, p. 158), the real-space R factor (Brändén & Jones, 1990), assessment of stereochemistry (Vriend, 1990; Laskowski, MacArthur, Moss & Thornton, 1993) and maximum-likelihood methods (Bricogne, 1984).

The most popular cross-validation evaluator in protein crystallography is the $R_{\rm free}$ factor (Brünger, 1992). This is to some extent analogous to full statistical cross-validation for assessing the capability of the model to reproduce the experimental results and to predict unmeasured data. The rationale in cross-validation methods is to split the complete data set into a training set, on which the model is built, and a test set, on which the model is tested. The procedure is repeated by considering, in turn, every data subset as the test set, and finally the global unbiased estimate of the model is obtained. It is often not possible to test the model against all the possible data subsets, as this requires unrealistic computing time. The compromise adopted is to evaluate the quality of the refinement on the basis of a randomly selected subset of the data. Use of $R_{\rm free}$ cross-validation in crystallography requires the omission of the reflections in the test set, for instance 10% randomly selected in the reciprocal space, Brünger (1992), from the refinement. In reality omission of about 1000 reflections is quite sufficient to provide a statistically meaningful sample. Omission of data in reciprocal space gives an unpredictable effect in real space. It reduces the convergence of the refinement and the quality of the refined model and introduces spurious features in the density map (Bacchi, Lamzin & Wilson, unpublished results).

2. Method

2.1. *Linear Hamilton R-factor test*

To preserve the completeness of the measured data, especially important in protein crystallography, a self-validation procedure is required. The question as to whether an improvement in R factor due to a decrease in the number of degrees of freedom is significant was first examined by Hamilton (1964, pp. 157–162; 1965), who formulated the problem as a linear hypothesis. Hamilton defined the R-factor ratio $R = R1/R2$, where $R1$ and $R2$ are the r.m.s. R factors referring to two stages of the refinement with different numbers of linear constraints. From the null hypothesis that the two refinements do not differ significantly,

$$R_{b,n-m,\alpha} = \{[b/(n-m)]\mathbf{F}_{b,n-m,\alpha} + 1\}^{1/2}, \qquad (1)$$

where $\mathbf{F}_{b,n-m,\alpha}$ denotes the $\mathbf{F}$-test analysis of the variance ratio for a b-dimensional linear hypothesis with $n-m$ degrees of freedom and a probability α of rejecting the hypothesis if the second refinement gives no advantage compared to the first one (type I error). Usually refinement proceeds from a more to a less constrained model, *i.e.* from less to more parameters, and the hypothesis that the releasing of restrictions really improves the model should be tested. Hamilton's analysis refers to unconstrained refinement of m parameters against n data, giving $n-m$ degrees of freedom. Introducing b linear constraints on the parameters leads to a higher number of degrees of freedom $(n-m+b)$. The two r.m.s. R factors, $R2$ and $R1$, are the relative estimated standard deviations of the distributions of the weighted $Fo-Fc$ deviates for the two refinements,

$$\text{r.m.s. } R = \{[\textstyle\sum w_i(Fo_i - Fc_i)^2]/[\sum w_i Fo_i^2]\}^{1/2}, \qquad (2)$$

The probability α that the observed r.m.s. R-factor ratio R expresses a significant improvement is,

$$P(\mathbf{F}_{b,n-m} = 1 - I_x[(n-m)/2, (b/2)], \qquad (3)$$

where $I_x[(n-m)/2, (b/2)]$ is the incomplete beta function and $x = [(n-m)/(n-m+b\mathbf{F})]$. The behaviour of the function depends strongly on the numerical values of $(n-m)/2$ and $(b/2)$, Fig. 1. If these are large, I_x approaches a step function centred at about $x = [(n-m+b)/(n-m)]$. This is always so in protein crystallography, and causes a sharp fall-off around the critical point $R = [(n-m+b)/(n-m)]^{1/2}$.

The Hamilton test requires a Gaussian distribution for the weighted deviates $(w_i)^{1/2}(Fo_i - Fc_i)$. This can be assumed if the residual $\sum w_i(Fo_i - Fc_i)^2$ is minimized by least squares. The distribution remains essentially Gaussian when the weighted residual in intensities rather than in amplitudes is minimized (as implemented in *SHELXL*93). Ideally $w_i = (1/\sigma_i^2)$ but in practice the weighting scheme used in crystallographic refinement is often derived from a synthesis of empirical considerations, program defaults and rules of thumb. Such modified weighting schemes are supposed to compensate for experimental uncertainties and the presence of systematic deviations in estimates of variances, particularly important for data from weakly diffracting crystals such as proteins. In terms of small-molecule crystallography

all protein data are weak. The weakness of purely statistical methods in the presence of systematic errors was clearly pointed out by Hamilton (1965) and the method proposed here is meant to be a guide to the crystallographer rather than an incontrovertible verdict.

2.2. *Extension for restrained refinement*

Hamilton limited his analysis to two refinements differing by the presence of b linear constraints. The more general case is now considered where the number of observations and the number of parameters change and restraints are used in a different manner.

Consider two refinements with different degrees of freedom, Df1 and Df2, and the condition that Df1 > Df2 and $R1 > R2$, which means that the first is more constrained and gives a higher r.m.s. R factor. We want to know if the improvement in r.m.s. R factor is significant and does not merely reflect a reduction of the number of degrees of freedom.

The number of degrees of freedom is defined as the number of observations minus the number of parameters. A linear constraint expresses an exact linear relationship between parameters as, for instance, $x/a = y/b$ for an atom located on a binary axis perpendicular to c in $P4_222$, or $U_{11} = U_{22} = U_{33}$ and $U_{12} = U_{23} = U_{13} = 0$ for isotropic refinement. In both cases introduction of the constraint lowers the number of parameters.

By means of prior knowledge of the chemical and physical behaviour of the system, a restraint introduces a condition that the system must obey within a certain degree of confidence expressed by a weighting coefficient. A restraint is an additional weighted observational equation. Either the introduction of N restraints or of N constraints results in an increase by N in the number of degrees of freedom of the refinement.

However, some restraints are redundant or are applied only if certain conditions arise (*e.g.* anti-bumping) and these cannot be easily counted. Therefore, we introduce a restraints completeness weighting coefficient w to define the effective number of observational equations,

$$N_{obs} = N_{refl} + wN_{restr}. \quad (4)$$

In the two limiting cases $w = 0$ corresponds to completely unrestrained refinement and $w = 1$ to a refinement where every restraint is treated as a full additional observation.

Let Nr1 and Nr2 be the number of reflections, $S1$ and $S2$ the number of restraints and $P1$ and $P2$ the number of parameters for the two refinements. The dimensionality (Dim) of the linear hypothesis is the difference between the number of degrees of freedom,

$$\begin{aligned} \text{Dim} &= \text{Df1} - \text{Df2} \\ &= (\text{Nr1} + w1 \cdot S1 - P1) - (\text{Nr2} + w2 \cdot S2 - P2). \end{aligned} \quad (5)$$

Hamilton's linear hypothesis refers to the particular case where Nr1 = Nr2, $P1 = P2$, $S2 = 0$, $w1 = 1$, $w2$ is indeterminate and Dim = $S1$.

Since we are considering the case where $R1 > R2$ when Df1 > Df2, the condition Dim > 0 must hold. This implies that $w1$ and $w2$ must satisfy the inequality,

$$w2 < [(\text{Nr1} - \text{Nr2} + \text{P2} - \text{P1})/S2] + w1(S1/S2). \quad (6)$$

In the two-dimensional space spanned by $w1$ and $w2$, a straight line with intercept $(\text{Nr1} - \text{Nr2} + P2 - P1)/S2$ and slope $S1/S2$ separates the weight-allowed area from the weight-forbidden area, Fig. 2. Since $S1/S2 \geq 0$, if $(\text{Nr1} - \text{Nr2} + P2 - P1)/S2 \geq 1$ there are no restrictions on $w1$ and $w2$. If the intercept is between 0 and 1, then the greater the value of $S1/S2$ (*i.e.* the less restrained is the second refinement compared to the first), the greater the allowed value of $w2$ for a given $w1$.

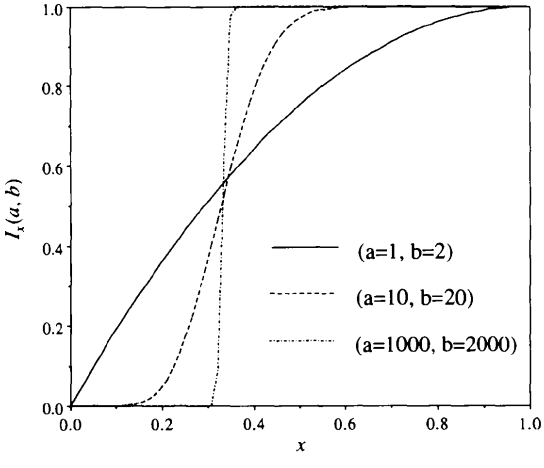

Fig. 1. Incomplete beta function $I_x(a,b)$ for different parameters.

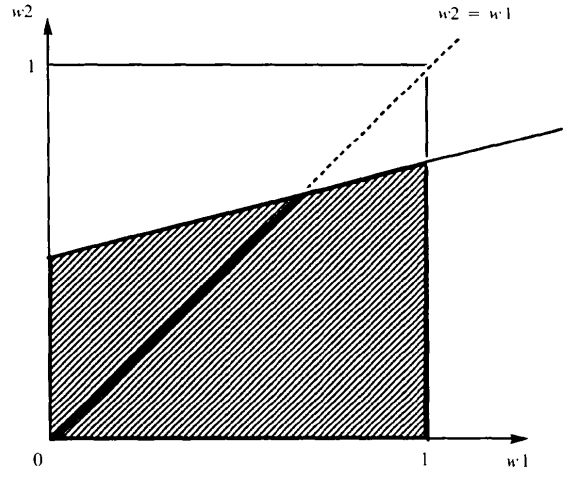

Fig. 2. Weights for restraints. The shaded area represents the points ($w1$,$w2$) which satisfy the assumption that the dimensionality must be positive.

Table 1. *Comparison of isotropic and anisotropic refinement for xylanase 1.5 Å*

	Reflections*	Parameters	Restraints	*R* factor† (%)	R.m.s. *R* factor‡ (%)
Isotropic	34460	7460	6450	14.0	20.3
Anisotropic	34460	17770	20610	10.2	14.9
R-factor ratio				1.37	1.36

*This corresponds to the working set (95%). The free set contained the remaining 1814 reflections (5%). †*R* factor = $(\sum |Fo_i - Fc_i|)/(\sum Fo_i)$. ‡R.m.s. *R* factor is defined in (2).

Under these assumptions, the probability that the improvement is significant can be calculated by a modification of (3),

$$P(\mathbf{F}_{\mathrm{Dim,\ Df2}}) = 1 - I_x[(\mathrm{Df2}/2),(\mathrm{Dim}/2)]. \quad (7)$$

The critical point is,

$$R = (\mathrm{Df1}/\mathrm{Df2})^{1/2}. \quad (8)$$

The improvement is only significant if the ratio between the two r.m.s. *R* factors is greater than the ratio between the numbers of degrees of freedom.

3. Application and discussion

3.1. *Evaluation of confidence*

The refinement of xylanase at 1.5 Å (Lamzin, Dauter, Dauter, Bisgard-Frantzen, Halkier & Wilson, to be published) using *SHELXL*93 (Sheldrick, 1993) is now considered. Table 1 summarizes the experimental data. The experimental distribution of weighted deviates $(w_i)^{1/2}(Fo_i - Fc_i)$ is Gaussian, Fig. 3.

Two refinements with isotropic and then anisotropic description of atomic thermal motion gave a ratio R = (r.m.s. R_{iso}/r.m.s. R_{aniso}) = 1.36. This value is very close to the ratio of the conventional *R* factors, Table 1, showing that here the Hamilton test could equally well be applied to weighted r.m.s *R* factors or to conventional unweighted *R* factors. The R_{free} factor improved to a smaller extent, from 16.6 to 13.5%, a ratio of 1.23. The number of reflections was equal for the two refinements.

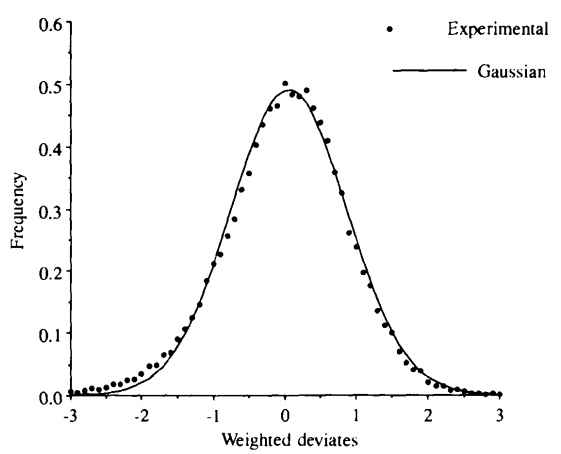

Fig. 3. Distribution of weighted deviates $(w_i)^{1/2}(Fo_i - Fc_i)$ for isotropic refinement of xylanase.

Since *w*1 and *w*2 are not known, as a first approximation they can be considered to be equal. Probability plots that the improvement is significant as a function of *R* for different but equal values of *w*1 and *w*2 are shown in Fig. 4. The anisotropic model is significantly better than the isotropic, as for any value of *w*1 = *w*2 the probability $P(R = 1.36)$ is essentially unity.

In the more general case, *w*2 must only obey inequality (6) and the probability is expressed as a function of *R*, *w*1 and *w*2. Fig. 5(*a*) shows that for $R = 1.36$ the improvement is significant for almost any value of *w*1 and *w*2. However, there is a very small region at the bottom of Fig. 5(*a*) with *w*2 close to zero, corresponding to an essentially unrestrained anisotropic model, where improvement in the r.m.s. *R* factor is not significant. Thus, at this resolution restraints on the anisotropic model are essential.

Suppose that a poorer ratio $R = 1.21$ ($R_{\mathrm{aniso}} = 11.6$, r.m.s. $R_{\mathrm{aniso}} = 17.0$) had been achieved, Fig. 5(*b*). This would be significant provided *w*1 is not lower than the threshold limit given by the borderline between

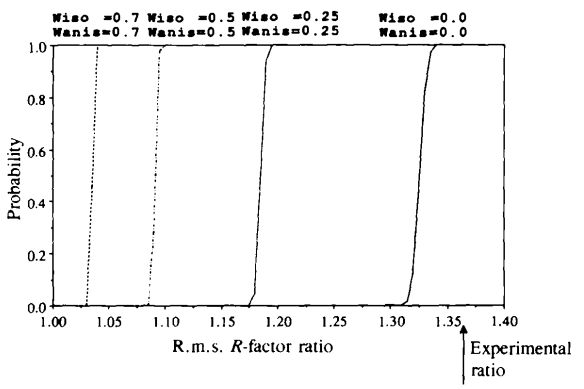

Fig. 4. Probability of a significant improvement in *R* factor for different values of *w*1 = *w*2 for anisotropic refinement of xylanase.

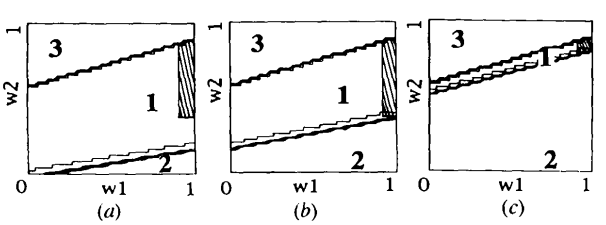

Fig. 5. Probability isolines (20, 40, 60, 80, 100%) of r.m.s. *R*-factor ratio, *R*, as a function of weighting coefficients in the case of xylanase: (*a*) for $R = 1.36$ (actual value obtained with anisotropic refinement); (*b*) for a hypothetically poorer improvement $R = 1.21$; (*c*) for $R = 1.03$. Label 1 indicates the region where the improvement is significant, 2 where it is not significant, 3 indicates impossible weighting schemes where inequality (6) does not hold. Shadowing indicates restrictions on *w*1 and *w*2 according to (10).

areas 1 and 2 in the plot. On further decrease of R, Fig. 5(*c*), $P(R)$ becomes more selective and for $R \simeq 1$ (*i.e.* r.m.s. $R_{aniso} \simeq$ r.m.s. R_{iso}) $P(R)$ is high only for weighting schemes such that Dim $\simeq 0$, where the number of degrees of freedom is equal for the two refinements. Under this condition the anisotropic model would be clearly shown not to be statistically meaningful.

3.2. *The role of w1 and w2*

As restraints are treated as extra observational equations they should be included as a sort of real-space contribution in the expression of the r.m.s. R factor. This contribution, compared to the diffraction term, is not straightforward to evaluate and depends on the unknown coefficient w. However, it can be neglected if the ratio between the effective number of restraints, $w{\cdot}S$, and the number of reflections, N_{refl}, is low. This is justified provided the allowed ranges of $w1$ and $w2$ are evaluated as described below.

The assumptions made by Hamilton (1965) may not all be satisfied in real life. Evaluation of the effective contribution of restraints can be related to problems in counting the number of degrees of freedom (Rogers, 1981). Introduction of the completeness coefficient w is aimed to take implicitly into account non-linearity, systematic errors, deviations from a normal distribution and uncertainty in the number and weights of the restraints. If for any point of the ($w1$,$w2$) space a high probability is observed, Fig. 5(*a*), the improvement in the model is significant even if Hamilton's hypotheses are not completely satisfied. If not, Fig. 5(*c*), the test is less robust and care should be taken in considering the results.

3.3. *Estimation of the completeness of restraints*

$w1$ and $w2$ can be estimated assuming that for a restrained model, the blind release of all restraints should not provide a significant improvement. Consider the refinement of a model M described by P parameters with N experimental observations and S restraints. We want to estimate the completeness w of the restraints, as defined in (3). A completely unrestrained refinement of M is carried out and it is assumed that the reduction in the r.m.s. R factor reflects only the reduction in the number of degrees of freedom. The extended Hamilton test can be applied in the form,

$$R_{critical} \geq R[\text{r.m.s. } R(\text{restrained})/\text{r.m.s. } R(\text{unrestrained})].$$

From (8),

$$R_{critical} = \{[\text{Df(restrained)}]/[\text{Df(unrestrained)}]\}^{1/2} = [(N-P+wS)/(N-P)]^{1/2}. \quad (9)$$

The limits for w are estimated as,

$$[(N-P)/S](R^2-1) \leq w \leq 1. \quad (10)$$

Table 2. *Estimation of completeness of restraints for refinement of xylanase at 1.5 Å*

	$R_{critical}$*	w_{min}*
Isotropic	1.11	0.95
Anisotropic	1.20	0.35

* $R_{critical}$ and w_{min} are defined in (9) and (10).

This provides an indication of the influence of the restraints on the refinement. A value of the lower limit for w, defined by the left-hand side of (10), close to or higher than 1 would indicate that too tight, or even incorrect, restraints have been applied. Their complete release produces a drop in the r.m.s. R factor higher than that expected from the decrease in Df alone. In other words if complete release of the restraints results in R greater than $R_{critical}$ from (9) for $w = 1$ the scheme of restraints may need to be reconsidered.

For xylanase the results are given in Table 2. Taking into account (9) the permitted region of two-dimensional ($w1$, $w2$) space is reduced. Fig. 5(*a*) shows that for the observed ratio R of 1.36 all weights permitted according to (9) give significant improvement, while this is not the case for hypothetical poorer R, Fig. 5(*b*) and 5(*c*). Fig. 5(*a*) clearly shows that satisfactory restraints have indeed been applied to the anisotropic model and that it is statistically reasonable, while in the isotropic case the restraints seem to have been too tight.

3.4. *Estimation of the expectation value for R_{free}*

Given that the free test set has been randomly selected, Cruickshank (personal communication) suggested that the expectation value for r.m.s. R_{free} can be derived from Hamilton (1964, p. 130, equation 51),

$$\text{r.m.s. } R_{free} = \text{r.m.s. } R[N_{obs}/(N_{obs}-N_{param})]^{1/2}. \quad (11)$$

If we define the number of observations as in (4) and (5), this gives,

$$\text{r.m.s. } R_{free} = \text{r.m.s. } R[(N+wS)/(N+wS-P)]^{1/2}. \quad (12)$$

If w is not known the two limiting values $w = 0$ and $w = 1$ can be considered to define the range in which the r.m.s. R_{free} should lie,

$$\begin{aligned} &\text{r.m.s. } R[(N+S)/(N+S-P)]^{1/2} \\ &\quad \leq \text{r.m.s. } R_{free} \\ &\quad \leq \text{r.m.s. } R[N/(N-P)]^{1/2}. \end{aligned} \quad (13)$$

The weights employed in the calculation of r.m.s. R_{free} must account only for the experimental errors in the diffraction data,

$$\text{r.m.s. } R_{free} = \{[\textstyle\sum(1/\sigma_i^2)(Fo_i - Fc_i)^2] \div [\sum(1/\sigma_i^2)Fo_i^2]\}^{1/2}. \quad (14)$$

For the isotropic refinement of xylanase the observed r.m.s. R_{free} was 22.5%, within the range 22.5–23.0% estimated from (13). For anisotropic refinement the observed value was 19.9%, again within the estimated range 18.2–21.5%. The expected drop in R_{free} can thus be predicted using Hamilton's analysis. This provides an additional means for estimating if the introduction of new parameters is statistically significant, *i.e.* results in the expected drop in R_{free}. Validation based on monitoring R_{free} alone is not able to reveal if an observed drop in R_{free} is significant.

4. Conclusions

Comparison between cross- and self-validation must take into account two factors: problems related to the omission of data and the demand of objectivity of the procedure. The use of R_{free} cross-validation simply moves the problem from the significance of a drop in the R factor to a drop in R_{free}. For xylanase the R_{free} for anisotropic refinement was lower than for isotropic but it was not obvious how much it should have fallen to demonstrate that the improvement was significant. If R_{free} cross-validation is used, then evaluation of the expectation value for the r.m.s. R_{free} is important to avoid the introduction of subjectivity and bias. The self-validation procedure described here, based on the Hamilton test, overcomes problems of omitting data and provides a more objective monitor. An intrinsic drawback in Hamilton's approach to validation is that it is based on a linear hypothesis, assumes Gaussian distribution for the deviates and is designed to work in the absence of systematic errors. Strictly speaking, not all crystallographic restraints are linear but an approximately linear behaviour can be assumed at least at the end of refinement.

Robustness of the test in the presence of systematic errors in the estimation of weights and of the resulting non-Gaussian distribution of the residuals is achieved by the introduction of the restraints completeness coefficient w, which damps the instability in the R parameter by varying the formal number of restraints. Examination of the probability that the improvement in the model is significant as a function of ($w1$, $w2$) indicates which ranges of weights are allowed for introduction of a new set of parameters. More general self-validation procedures for protein crystallography, which unlike the Hamilton test do not demand Gaussian distribution of deviates, will be considered in future work.

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