Effect of High Concentration of Inert Cosolutes on the Refolding of an Enzyme

CARBONIC ANHYDRASE B IN SUCROSE AND FICOLL 70*

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The kinetics of refolding of carbonic anhydrase II following transfer from a buffer containing 5 M guanidinium chloride to a buffer containing 0.5 M guanidinium chloride were studied by measuring the time-dependent recovery of enzymatic activity. Experiments were carried out in buffer containing concentrations of two “inert” cosolutes, sucrose and Ficoll 70, a sucrose polymer, at concentrations up to 150 g/liter. Data analysis indicates that both cosolutes significantly accelerate the rate of refolding to native or compact near-native conformations, but decrease the fraction of catalytically active enzyme recovered in the limit of long time. According to the simplest model that fits the data, both cosolutes accelerate a competing side reaction yielding inactive compact species. Acceleration of the side reaction by Ficoll is significantly greater than that of sucrose at equal w/v concentrations.

It is now becoming more widely recognized that excluded volume effects arising from the presence of a high total concentration of macromolecules in almost all biological fluids may significantly affect both the conformation and association state of each species of macromolecule in the fluid, whether dilute or concentrated, in an entirely nonspecific manner (1–3). Some years ago it was suggested on theoretical grounds that intermolecular excluded volume in a highly volume-occupied solution (termed “macromolecular crowding”) could stabilize the compact state relative to any less compact non-native state of a protein at equilibrium (4–6). A number of experimental studies have qualitatively, and in some cases quantitatively confirmed this prediction (7, 8). However, the effect of crowding upon the kinetics of protein isomerization is less predictable, as such effects depend in principle upon details of a particular reaction pathway (for example, the conformation of a transition state) that are for the most part unknown.

Studies of the effects of macromolecular crowding on the rates of refolding of several denatured proteins, including reduced lysozyme (9), glucose-6-phosphate dehydrogenase and protein-disulfide isomerase (10), geralddehyde-3-phosphate dehydrogenase (11), and GroEL (12), following reexposure to native-favoring conditions, have been reported. As noted earlier (13), with the exception of lysozyme, all of the other proteins listed are homo-oligomers in the native state. Hence regain of native structure or enzymatic activity is a complex process involving refolding and association of polypeptide chains, both of which are subject to crowding effects (6, 14–20). In addition, enzymatic activity was only partially regained in the presence of “inert” crowding agents, indicating the presence of misfolding and/or aggregation, neither of which was taken into account in interpreting the data.

To circumvent some of the difficulties associated with interpretation of the results of prior studies, we have studied the effect of a neutral “inert” polymer, Ficoll 70, on kinetics of the recovery of enzymatic activity of a simple protein, carbonic anhydrase B (CAB,2 30 kDa) that is a monomer in solution and lacks disulfide cross-links. Ficoll 70 was selected as a macromolecular crowding agent because it is a highly cross-linked copolymer of sucrose and epichlorhydrin that is significantly more compact than linear polymers, so the viscosity of highly concentrated solutions remains substantially lower than that of comparable linear polymers (for example, dextrans) at the same w/v concentration. To more clearly delineate the role of cosolute size we have also conducted a parallel study of the effect of sucrose, the “monomer” of Ficoll, on the rate of refolding of carbonic anhydrase.

MATERIALS AND METHODS

Bovine carbonic anhydrase B (CAB) was purchased from Biozyme Laboratories (London, England) and used without further purification. Protein concentration was spectrophotometrically measured using an extinction coefficient at 280 nm of 4.5 × 10^4 M⁻¹ cm⁻¹ for native carbonic anhydrase (manufacturer’s specification) and a calculated 3.8 × 10^4 M⁻¹ cm⁻¹ for denatured CAB. Ficoll 70 and p-nitrophenylacetate (pNPA) were obtained from Sigma and 100% acetonitrile (Pierce) was used to prepare 20 mM stock solutions of the substrate that were kept at 4 °C and always used within 10 days of preparation. Histidine and dipicolinic acid were from ICN and Sigma, respectively. Dilution of the substrate into dilution buffers for measurements was made just before use, yielding a final acetonitrile concentration of 0.16% which does not affect the pH of the solution (21) or the structure and activity of carbonic anhydrase (22). Sucrose and guanidinium chloride (GuHCl) were from Invitrogen. Solu-

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2 The abbreviations used are: CAB, bovine carbonic anhydrase B; GuHCl, guanidinium chloride; pNPA, p-nitrophenylacetate, substrate for the esterase reaction catalyzed by carbonic anhydrase; PNP, p-nitrophenolate ion, hydrolysis product arising from the degradation of the substrate.
tions of the protein, substrate, denaturant and crowders were prepared by dilution into 0.1 M Tris-HCl buffer (ICN), pH 7.5, containing Ficoll 70 or sucrose as required. The increase in solution viscosity arising from addition of Ficoll 70 to a final concentration of 150 g/liter is around 15%.

Methods

CD Spectroscopy—CAB spectra in the near and far UV-CD spectra were recorded at around 17 μM and 3.5 μM, respectively, at room temperature using a Jasco J-715 spectropolarimeter, using 1 cm and 0.1 cm path length quartz cells and a scan rate of 50 nm/min. Addition of Ficoll 70 impaired in all cases data acquisition below 205 nm. The buffer signal was subtracted from the experimental data, and the corrected ellipticities were converted into mean residue ellipticities [θ] using a mean molecular mass per residue of 112 for carbonic anhydrase (Mw 29047.87 calculated from amino acid sequence, 259 residues).

Fluorescence Spectroscopy—Emission spectra of native and denatured carbonic anhydrase (excited at 280 nm) were recorded at a protein concentration of around 4.5 μM at 25 °C in a PTI Quantamaster spectrofluorometer using 1-cm path length quartz cuvettes. An intensity control with quinine was performed daily. Fluorescence intensities were corrected by substraction of blanks. Solutions were manually mixed before data collection.

Time-dependent Activity Measurements—The irreversible hydrolysis of the synthetic substrate p-NPA to the colored product p-nitrophenolate ion (pNP) was monitored spectrophotometrically by absorbance at 400 nm, a wavelength at which neither substrate nor cosolute absorb.

CAB was incubated in a denaturing buffer containing 5 M GuHCl for around 1 h. Shortly before completion of incubation, a stock solution of substrate was diluted into denaturing buffers containing different concentrations of Ficoll 70 or sucrose and 99 μl of denaturing buffer loaded into individual wells of a 96-well half-area plate. To initiate the refolding reaction simultaneously in all wells, 1 μl of the protein solution (∼33 μM) was placed on the wall of each well above the substrate solution, and mixing of enzyme and substrate achieved by centrifuging the plate for 5 min at 4000 rpm. Absorbance of each well at 400 nm was then read at 1-min intervals over a period of 4 h in a Spectramax 250 or Spectramax 190 spectrophotometric plate reader (Molecular Devices). The contents of the microplate were mixed for 3 s prior to each absorbance reading using the automatic shaking option of the plate reader. The final 100-μl reaction volume in each well had a calculated optical path length of 5.27 ± 0.01 mm. Refolding experiments and controls with native protein and baseline under the same conditions were always performed in duplicate, and the time dependence of mean absorbance was analyzed as described below after normalizing to 1 cm path length.

Analysis of Time-dependent Absorbance Measurements—Preliminary experiments established that absorbance at 400 nm is proportional to the concentration of the product p-NPA generated by hydrolysis of substrate p-NPA. Thus the time-dependence of absorbance may be written as Equation 1,

\[ A(t) = A_0 + \alpha [P]_t \]  (Eq. 1)

where \( A_0 \) denotes the baseline absorbance, \( \alpha \) is an extinction coefficient, and \([P]_t\) the concentration of product. It follows that Equation 2,

\[ \frac{d[P]}{dt} = \frac{dA^*}{dt} \]  (Eq. 2)

where \( A^* \) denotes the baseline-corrected absorbance \( A - A_0 \) and \( \beta = 1/\alpha \).

The hydrolysis of substrate may occur spontaneously or be catalyzed by enzyme. Thus we obtain the mass action rate expression in Equation 3,

\[ \frac{d[P]}{dt} = (k_b + k_s[E])[S] \]  (Eq. 3)

where \( k_b \) denotes a pseudo-first-order rate constant for spontaneous (“background”) hydrolysis, and \( k_s \) denotes an effective second-order rate constant for enzyme-catalyzed hydrolysis, assuming that the enzyme obeys Michaelis-Menten kinetics, i.e., 

\( k_e = k_{cat}/K_m \). We further define the quantity \( f_A \) as the fraction of total enzyme \( E_{TOT} \) that is catalytically active. Equation 4 follows.

\[ \frac{d[P]}{dt} = (k_b + k_E E_{TOT} f_A)[S] \]  (Eq. 4)

This equation may be solved to yield Equation 5,

\[ A^*(t) = A^*_{max} \left( 1 - \exp \left[ J(t) \right] \right) \]  (Eq. 5)

where \( A^*_{max} \) is the baseline-corrected absorbance corresponding to the stoichiometric conversion of all substrate to product (=\( \alpha S_{iso} \)), and Equation 6.

\[ J(t) = -k_b t - k_E E_{TOT} \int_{0}^{t} f_A dt \]  (Eq. 6)

Recovery of enzyme activity is modeled by an exponential Equation 7,

\[ f_A(t) = f_a \left( 1 - e^{-\nu t} \right) \]  (Eq. 7)

where \( f_a \) denotes the fraction of active protein in the limit of long time and \( t_f \) the relaxation time for refolding. Equation 8 follows.

\[ \int_{0}^{t} f_A dt = f_a (t - t_f \left( 1 - e^{-\nu t_f} \right)) \]  (Eq. 8)

Although the time-dependent recovery of activity could in principle be more complex than the simple exponential indicated in Equation 7, it is demonstrated below that the use of a single exponential to describe the time dependence of \( f_A \) is sufficient to describe our results to within experimental uncertainty.
Inert Cosolutes Affect Rate and Extent of CAB Refolding

**Inert Cosolutes Affect Rate and Extent of CAB Refolding**

Selection of Conditions for Renaturation Experiments—CAB was incubated at several guanidinium chloride concentrations ranging from 0 to 6 M. At 2 M denaturant concentration the minimum in ellipticity at around 210 nm is lost, and there is a progressive loss in signal up to 5 M GuHCl. The measured minimum in ellipticity at around 210 nm is lost, and there is a
ing us to discard the occurrence of significant aggregation on the time scale of refolding.

**Effect of Ficoll and Sucrose on the Spectroscopic Characteristics of CAB**—The effect of Ficoll on the structural integrity of carbonic anhydrase was examined by comparing the CD spectra of native carbonic anhydrase in the absence and presence of Ficoll 70 (dashed lines) or sucrose (solid lines). Carbonic anhydrase concentrations were 3.5 μM and 27.5 μM for far- and near-CD measurements, respectively. Cand D, emission fluorescence spectra of native and denatured CAB, respectively, in the absence (solid) and presence of 150 g/liter Ficoll (dashed). Carbonic anhydrase concentration used was 6.2 μM.

**RESULTS**

Effect of Carbonic Anhydrase Concentration on the Kinetics of Regain of Enzymatic Activity—The effect of enzyme concentration on the refolding pathway was checked by varying the protein concentration between 0.11 and 1 μM. Analysis of the data by Equations 5, 6, and 7 gave comparable results for the fitting parameters in the three cases, except for the expected variation in the $k_{e,\bar{F}_{TOT}}$ value. There was no significant effect on $f_e$ or $t_e$ with the increase in protein concentration (not shown), allow-
Inert Cosolutes Affect Rate and Extent of CAB Refolding

with maximum shifted to 351 nm and lower intensity. Ficoll 70 has no effect upon the fluorescence of the unfolded protein and only a slight diminishment of the intensity of native carbonic anhydrase fluorescence, which is not indicative of any major effect upon native structure. There was no significant difference between the spectrum of the native protein recorded just after its dilution into the Ficoll 70 solution and that of carbonic anhydrase previously stabilized in the crowder solutions (not shown), allowing us to neglect nonspecific effects arising from the dilution of the protein into the crowder.

Effect of Ficoll and Sucrose on the Kinetics of Regain of Enzymatic Activity—In Fig. 3, A and B are plotted time-dependent absorbance profiles reflecting production of pNP, the product of both spontaneous and enzyme-catalyzed hydrolysis of pNPA, in the absence of enzyme (triangle), in the presence of native enzyme (circles), and in the presence of refolding enzyme (squares) in the absence and presence of sucrose (Fig. 3A) and Ficoll (Fig. 3B) at 50 and 150 g/liter. Comparable data were obtained at cosolute concentrations of 25, 75, and 100 g/liter (not shown). It is evident that the rate of non-enzymatic hydrolysis of pNPA is increased with increasing concentrations of both sucrose and Ficoll, the effect of sucrose being larger. The reduced difference between the product formation curves in the absence and presence of native enzyme leads to increased uncertainty in the analysis of the product formation curve obtained for refolding protein, as detailed below.

Analysis of these profiles described in detail under “Discussion” reveals that the refolding protein does not fully regain native enzymatic activity even in the limit of long time, in qualitative accord with the results of an earlier study (25). This result indicates the presence of one or more side reactions leading to non-native products, some of which could involve aggregation of the refolding protein. However, two experimental facts indicate the absence of significant aggregation accompanying protein refolding at the highly dilute protein concentration used in our activity measurements: (i) no time-dependent turbidity was detectable at 330 nm at any Ficoll concentration and (ii) there was no effect of protein concentration on the kinetics of refolding.

For a given set of experimental conditions (i.e. concentration of cosolute) all three product accumulation curves were modeled globally using Equations 5, 6, and 8 to obtain best-fit values of the parameters $k_p$, $k_{E_{TOT}}$, $f_\infty$, and $t_f$. The product accumulation curves calculated using best-fit parameter values obtained by this modeling procedure, which are plotted together with the data to within the precision of measurement, and in almost all cases the distribution of residuals is random.

The mean and 95% confidence limits (when determinable) of the best-fit parameter values obtained in 5–20 replicate measurements performed at each Ficoll and sucrose concentration are tabulated in Table 1 and the values of $t_f$ and $f_\infty$ plotted against cosolute concentration in Fig. 4, A and B. Best-fit linear regression lines are also plotted in this figure. The presence of increasing concentrations of Ficoll 70 or sucrose results in a decrease in the total amount of active protein recovered and an apparent decrease in the decay time of refolding that becomes immeasurably small at high additive concentrations because most of the refolding appears to take place within the dead time of our measurement ($\approx 6.5$ min). The mean best-fit values of $t_f$ and $f_\infty$ at each cosolute concentration were used to calculate the fractional recovery as a function of time according to Equation 7, and these recovery curves are plotted in Fig. 5. There is a decrease in the value of $k_{E_{TOT}}$ estimated from the native control with increasing concentrations of cosolute. The fact that similar CD spectra are obtained in the presence of different Ficoll and sucrose concentrations (Fig. 2) suggests that the slowing in the reaction rate observed in the native activity is not due to any change in the native structure of the protein although, as previously stated, this possibility cannot be com-

3 This was accomplished by including in our model the condition that $k_{E_{TOT}} = 0$ for the data set obtained in the absence of enzyme and $f_\infty(t) = 1$ for all $t$ for the data set obtained with native enzyme.

4 The increased rate of non-enzymatic hydrolysis of pNPA in the presence of high cosolute concentrations also contributes to a loss of experimental resolution, as mentioned above.
**Inert Cosolutes Affect Rate and Extent of CAB Refolding**

**TABLE 1**

Effect of cosolutes on the kinetic parameters obtained by fitting time-dependent absorbance profiles of CAB

| Additive     | $A_{0b}$ | $A_{0n}$ | $A_{0r}$ | $A_{max}$ | $k_b$ | $k_{E_{TOT}}$ | $f_\infty$ | log $t_f$ |
|--------------|----------|----------|----------|-----------|-------|---------------|------------|-----------|
| No additive  | 0.084 ± 0.002 | 0.092 ± 0.004 | 0.089 ± 0.002 | 0.625 ± 0.016 | 0.0021 ± 6.94 $10^{-5}$ | 0.0043 ± 0.0002 | 0.701 ± 0.022 | 1.05 ± 0.04 |
| Ficoll 70    | 25 g/liter | 0.089 ± 0.004 | 0.096 ± 0.004 | 0.093 ± 0.004 | 0.629 ± 0.010 | 0.0024 ± 7.50 $10^{-5}$ | 0.0047 ± 9.98 $10^{-5}$ | 0.638 ± 0.042 | 0.74 ± 0.20 |
|              | 50 g/liter | 0.097 ± 0.002 | 0.102 ± 0.002 | 0.103 ± 0.002 | 0.618 ± 0.012 | 0.0027 ± 4.06 $10^{-5}$ | 0.0043 ± 0.0002 | 0.636 ± 0.048 | 0.83 ± 0.12 |
|              | 75 g/liter | 0.102 ± 0.004 | 0.110 ± 0.004 | 0.108 ± 0.004 | 0.631 ± 0.012 | 0.0029 ± 14.7 $10^{-5}$ | 0.0043 ± 0.0004 | 0.509 ± 0.048 | <0.86 |
|              | 100 g/liter | 0.106 ± 0.004 | 0.111 ± 0.004 | 0.113 ± 0.006 | 0.611 ± 0.018 | 0.0031 ± 15.3 $10^{-5}$ | 0.0039 ± 0.0004 | 0.548 ± 0.038 | <0.91 |
|              | 150 g/liter | 0.110 ± 0.006 | 0.118 ± 0.006 | 0.119 ± 0.006 | 0.611 ± 0.026 | 0.0036 ± 0.0002 | 0.0034 ± 0.0006 | 0.467 ± 0.070 | <0.96 |
| Sucrose      | 25 g/liter | 0.090 ± 0.004 | 0.095 ± 0.002 | 0.096 ± 0.004 | 0.659 ± 0.056 | 0.0029 ± 15.1 $10^{-5}$ | 0.0047 ± 0.0008 | 0.721 ± 0.082 | 0.95 ± 0.10 |
|              | 50 g/liter | 0.095 ± 0.002 | 0.102 ± 0.002 | 0.102 ± 0.002 | 0.648 ± 0.026 | 0.0036 ± 9.06 $10^{-5}$ | 0.0045 ± 19.2 $10^{-5}$ | 0.664 ± 0.032 | <1.04 |
|              | 75 g/liter | 0.098 ± 0.006 | 0.106 ± 0.006 | 0.109 ± 0.008 | 0.654 ± 0.034 | 0.0042 ± 0.0004 | 0.0037 ± 0.0012 | 0.719 ± 0.102 | 0.78 ± 0.14 |
|              | 100 g/liter | 0.105 ± 0.004 | 0.114 ± 0.002 | 0.113 ± 0.002 | 0.652 ± 0.032 | 0.0052 ± 19.1 $10^{-5}$ | 0.0042 ± 0.0004 | 0.612 ± 0.054 | <0.99 |
|              | 150 g/liter | 0.109 ± 0.012 | 0.118 ± 0.012 | 0.119 ± 0.010 | 0.627 ± 0.024 | 0.0062 ± 0.0006 | 0.0031 ± 0.0012 | 0.610 ± 0.132 | <0.74 |

**FIGURE 4.** Change in the decay time of refolding and fractional recovery of native enzyme with cosolutes. Effect of Ficoll 70 (circles) and sucrose (triangles) on $t_f$, normalized regarding the value in the absence of cosolute (A) and fractional recovery of carbonic anhydrase (B). Symbols are used only when upper and lower confidence limits were well determined. Solid straight lines are the linear fittings to the experimental data and dashed lines in A connect experimental data points. Error bars correspond to two times the standard deviation of the mean. Each data point is the average of at least five different measurements, which were performed in turn in duplicate.

**FIGURE 5.** Simulation of the fractional activity recovered with time. Fractional recovery was calculated according to Equation 7 using the mean values obtained by fitting of the exponential model to all the experimental data (Table 1) in the absence (solid lines) and presence of Ficoll 70 (A) or sucrose (B) at 50 g/liter (dashed) and 150 g/liter (dotted). The vertical dashed lines delineate the range of times over which data were collected (~4 h), while short times on the left correspond to the delay time of mixing previous to the collection of data.

Our findings dictate that following the introduction of unfolded protein into a buffer favoring refolding, there must be at least one side reaction leading to the formation of some frac-

hindered access of substrate to the active site of the enzyme, as previously suggested for other enzymes and crowding agents (26).

**DISCUSSION**

Our findings dictate that following the introduction of unfolded protein into a buffer favoring refolding, there must be at least one side reaction leading to the formation of some frac-

Completely ruled out. Because $E_{TOT}$ is constant and only small changes due to experimental error can be expected, this effect could be accounted for by the hypothesis that the cosolutes hinder access of substrate to the active site of the enzyme, as previously suggested for other enzymes and crowding agents (26).
tion of catalytically inactive protein. The following models thus represent possible molecular mechanisms that we have explored. They are listed in order of increasing complexity.

Model I: Parallel Refolding—It is assumed that the unfolded protein U may either refold to the correct catalytically active conformation A, or one of a manifold of closely related compact and near-native conformations which are however catalytically inactive. The sum of all of non-active conformations can be grouped into a single inactive conformation I as shown in Reaction Scheme 1.

\[
\begin{align*}
U & \rightarrow A \quad (k_A) \\
U & \rightarrow I \quad (k_I)
\end{align*}
\]

REACTION SCHEME 1

Solution of the rate equations described by this model leads to a simple analytical expression for the recovery curve (Equation 9),

\[
f_A(t) = \frac{k_A}{k_A + k_I} \left[ 1 - e^{-(k_A + k_I) t} \right] \quad (Eq. 9)
\]

which is equivalent to the empirical model used to fit the data using Equation 7, with parameter substitutions given by Equations 10 and 11.

\[
f_a = \frac{k_A}{k_A + k_I} \quad (Eq. 10)
\]

and

\[
t_f = \frac{1}{k_A + k_I} \quad (Eq. 11)
\]

Model II: Parallel Refolding with Zinc Rebinding—This model is based upon the assumption that the zinc ion normally bound to native carbonic anhydrase (27) dissociates upon exposure to 5 M GuHCl, and that rebinding of Zn\(^{2+}\) is a necessary prerequisite to reacquisition of catalytic activity shown in Reaction Scheme 2.

\[
\begin{align*}
U + Zn & \rightarrow A \quad (k_A) \\
U & \rightarrow I \quad (k_I)
\end{align*}
\]

REACTION SCHEME 2

This model predicts that both the relaxation time for refolding and the long time limit of fractional activity regain would be highly dependent upon the concentration of free zinc in the refolding buffer. In control experiments, we changed the amount of free zinc in the refolding buffer by adding either a large excess of ZnCl\(_2\) or by adding a large excess of a zinc chelator (either histidine or dipicolinic acid). Neither modification of the experimental protocol resulted in a measurable change in activity regain kinetics, in qualitative agreement with results obtained in a prior study of the effect of zinc on carbonic anhydrase refolding (28), demonstrating that this model need not be considered further.

Model III: Two Kinetically Distinct Populations of Unfolded Conformations—Slow stages in refolding are usually associated with the isomerization of essential (protected) prolines. Carbonic anhydrase has 19 prolines (27), 4 of them non-exposed to the solvent that could be, in principle, the ones determining the acquisition of the proper native state. A group of conformations containing rate-limiting non-native proline isomer(s) might be expected to refold significantly more slowly than those retaining the native-like proline isomer. The simplest model of this type follows: Let \(U_1\) contain native-like proline isomers and \(U_2\) contain non-native proline isomers (Reaction Scheme 3),

\[
\begin{align*}
U_1 & \rightarrow A \quad (k_{U1A}) \\
U_2 & \rightarrow I \quad (k_{U2I})
\end{align*}
\]

where \(k_{U2I}\) is a measure of the (average) rate of proline isomerization and is expected to be much smaller than \(k_{U1A}\). The corresponding rate equations were solved numerically and used to model our data. We found that we could fit our data satisfactorily, but four adjustable parameters were required (3 rate constants plus the fractional abundance of \(U_1\) at zero time), which is two more than required in Model I, and the uncertainty of best-fit parameter determination was unacceptably great. If, in the future, the dead time between dilution of enzyme into refolding buffer and the initiation of data acquisition can be substantially reduced, data obtained at significantly shorter elapsed times may provide the increased resolution required to distinguish between this model and Model I, and to evaluate the additional parameters invoked in this model.

Model IV: Inactivation via Aggregation—The following Reaction Scheme 4 is the simplest model of this type.

\[
\begin{align*}
U & \rightarrow A \quad (k_A) \\
U + U & \rightarrow I \quad (k_I)
\end{align*}
\]

REACTION SCHEME 4

This model differs from Model I in that \(k_I\) is a second-order rate constant instead of a first-order rate constant. Model IV predicts that the rate and extent of inactivation should increase substantially with increasing enzyme concentration, which conflicts with the results of control experiments described above, and hence was eliminated from further consideration.

Model I was found to be the simplest model capable of accounting for our combined data quantitatively, and we adopt it as a provisional mechanistic description of refolding kinetics. We may solve Equations 10 and 11 simultaneously to calculate the rate constants \(k_A\) and \(k_I\) as functions of the empirical parameters \(f_a\) and \(t_f\) shown in Equations 12 and 13.

\[
k_A = \frac{f_a}{t_f} \quad (Eq. 12)
\]

\[
k_I = \frac{1 - f_a}{t_f} \quad (Eq. 13)
\]

In Fig. 6, we plot the estimated dependence of \(k_A\) and \(k_I\) upon the concentrations of sucrose and Ficoll, calculated using values of \(f_a\) and \(t_f\) obtained from the regression lines plotted in Fig. 4, A and B. According to the model, the highest concentrations of both sucrose and Ficoll result in a roughly 2-fold increase in
Inert Cosolutes Affect Rate and Extent of CAB Refolding

| FIGURE 6. Calculation of the effect of cosolutes on the reaction rates for refolding assuming Model I. Variation of the reaction rates \( k_A \) (dashed) and \( k_I \) (solid line) with the concentration of Ficoll 70 or sucrose (1 or 2, respectively). |

\( k_A \), the highest concentration of sucrose results in a roughly 3-fold increase in \( k_A \), and the highest concentration of Ficoll results in a roughly 6-fold increase in \( k_I \). Why should Ficoll more strongly promote refolding to an inactive species than sucrose? We have no definite answer, but we do know that smaller volume excluding cosolutes are more sensitive to details of the conformation of a test macromolecule than larger volume excluding cosolutes, as they can probe the surface of the test molecule with higher resolution (3). Therefore we speculate that while both cosolutes enhance isomerizations that result in more compact conformations, in qualitative accord with the predictions of excluded volume theory (4, 5) the smaller cosolute may be more sensitive to small differences in excluded volume that might distinguish the globally most compact nearly native (catalytically active) conformation from a large set of compact nearly native, but catalytically inactive, conformations.

Prior studies of refolding have shown that crowding can contribute to a loss of enzymatic activity through enhancement of side reactions leading to aggregation (9, 29). The present study was carried out using concentrations of enzyme so low that significant aggregation did not take place on the time scale of our experiments. Thus we could demonstrate unambiguously that crowding can accelerate refolding of a monomeric protein not only to its native, enzymatically active conformation, but also to compact non-native conformations that are similar in overall structure but lacking enzymatic activity.

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