Kinetics and Mechanism of Refolding of Bovine Carbonic Anhydrase. A Probe Study of the Formation of the Active Site†

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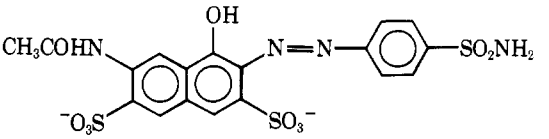
ABSTRACT: In kinetic studies of the folding of bovine carbonic anhydrase from disorganized to native structure, an azosulfonamide, 2-(4-sulfomylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (I), has been used as a probe to follow the dynamics of formation of the active site region. The probe is a specific inhibitor of the native enzyme that binds in the active site crevice. The experiments, with previous data (Yazgan, A., and Henkens, R. W. (1972), *Biochemistry 11*, 1314), show that a tight binding site for I forms at an intermediate stage in the folding process. A subsequent conformational change perturbs the visible absorption and circular dichroism of bound I and could result in even tighter binding. The subsequent change completes formation of the active site. This is shown by results from separate experiments on the kinetics of recovery of activity (*p*-nitrophenyl acetate as substrate). Similar probe methods could be used with other proteins and enzymes to study the kinetics and mechanism of regeneration of specific sites—for example, the active site.

Like any complex reaction, the folding of a disordered polypeptide to form an organized native protein must occur in a number of steps. And yet, in most early studies, the reactions appeared to be two-state transitions. This simply means that all partially folded molecules are very unstable. In such cases, it is virtually impossible to obtain any information on the nature of the partially folded intermediates, or on the mechanism of protein folding. During the last 4 or 5 years, less cooperative protein folding reactions involving detectable intermediates have been found (for recent reviews, see Wetlaufer and Ristow, 1973; Baldwin, 1975; Anfinsen and Scheraga, 1975). To determine folding mechanisms, the next logical step is to determine the structure of the transient kinetic intermediates.

Several groups have reported studies on the refolding of human and bovine carbonic anhydrases denatured by guanidine hydrochloride, Gdn·HCl[1] (Yazgan and Henkens, 1972; Wong and Tanford, 1973; Carlsson et al., 1973, 1975; Wong and Hamlin, 1975). The fully Gdn·HCl denatured bovine enzyme behaves as a randomly coiled polypeptide (Wong and Tanford, 1973). The zinc ion, however, remains coordinated to the unfolded enzyme (Stein and Henkens, unpublished), possibly to the chelate formed by His-93 and His-95, and influences the pathway of the folding reaction (Yazgan and Henkens, 1972; Wong and Hamlin, 1975). Intermediates in the folding process have been detected. These have been more fully characterized for the human B enzyme (Carlsson et al., 1975), but the rate and extent of refolding of this enzyme depends strongly on protein concentration, and good yields of regenerated enzyme are obtained only at very low protein concentrations. It is clear from this that the bovine enzyme is more suitable for model studies on the kinetics and mechanism of protein folding.

Our aim in studying the refolding of bovine carbonic anhydrase denatured by Gdn·HCl is to be able to follow the structural dynamics of the complete folding process—from randomly coiled polypeptide to native protein—using a number of methods. Here we report a probe study of the dynamics of formation of the binding site for a sulfonamide inhibitor, I, and



I

results on the kinetics of recovery of activity. The studies show that a tight binding site for the specific inhibitor (Coleman, 1967, 1968) forms at an intermediate stage in the folding process, before the active site is fully regenerated.

Materials and Methods

Materials. Compound I was purchased from Winthrop Laboratories, New York, N.Y., and purified by recrystallization from methanol–water, or chromatography on Florisil obtained from Fisher Scientific Co. (for details see, Ko, 1974). Stock solutions were stored in the dark.

Bovine carbonic anhydrase, obtained as a lyophilized material from Worthington Biochemical Corp., was purified by chromatography on DEAE-Sephadex A-25 (Yeagle et al., 1975). Protein concentrations were determined from absorbance at 280 nm, using a molar absorptivity of 5.7×10^4 M^{-1} cm^{-1} (Lindskog and Nyman, 1964).

Guanidine hydrochloride was purchased from Heico, Inc., Delaware Water Gap, Pa. Stock solutions were passed through a fritted glass filter and used without further purification. The ultraviolet absorption, pH, and melting point met published criteria for purity (Wong et al., 1971). Gdn·HCl concentrations were determined from the refractive index (Nozaki, 1972) measured on an Abbe refractometer.

Ultrapure Tris was purchased from Schwarz/Mann, Orangeburg, N.Y., and used without further purification. Tris–sulfate buffers were prepared by adjusting the pH of solutions of Tris with reagent grade sulfuric acid.

Deionized water provided by Continental Water Service was filtered through a 0.45-μm Gelman filter and used to wash glassware and prepare all aqueous solutions.

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[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; CD, circular dichroism.

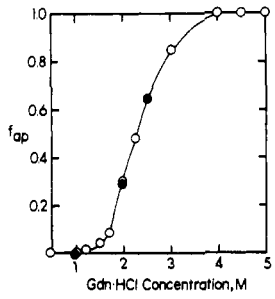

FIGURE 1: Equilibrium denaturation and renaturation transition of the enzyme–I complex. The transition was monitored by changes in the absorbance of I at 545 nm; f_{ap} is the apparent extent of denaturation, given by eq 1 in text. Open circles are for denaturation; filled circles are for renaturation.

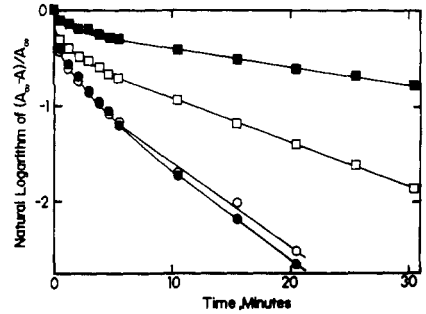

FIGURE 2: Kinetics of change in the absorbance of I at 545 nm during enzyme refolding. Absorbance measurements made relative to a reference lacking the enzyme but the same in other respects; the initial absorbance is zero. Kinetics described by eq 2 in text: (■) 1.92 M Gdn·HCl, pH 8.4; (□) 1.50 M Gdn·HCl, pH 8.4; (○) 1.24 M Gdn·HCl, pH 8.4; (●) 1.00 M Gdn·HCl, pH 6.0 (see experimental methods for additional information).

Methods. Absorbance measurements were made with a Cary 15 spectrophotometer, periodically checked for absorbance linearity, wavelength calibration, and excessive stray light by Aviv Associates, Lakewood, N.J. CD measurements were made with a Durrum-Jasco ORD/UV/CD-5 modified by Sproul Scientific Instruments, Tucson, Ariz., to improve the signal-to-noise ratio. For CD measurements, total absorbance in the region of interest was always less than 0.5.

Binding of I to carbonic anhydrase results in changes in its visible absorption (difference spectrum extrema at 545 and 485 nm). Also binding of I to carbonic anhydrase induces dissymmetry into electronic transitions of I and gives rise to visible CD bands (Coleman, 1968). These optical effects have been used to detect changes in the association of I with the enzyme. Experiments that will be discussed in the next section show that I can be used as a probe to report the dynamics of formation of its own specific binding site. The experimental method is simply to monitor optical changes in added I during refolding of the Gdn·HCl denatured enzyme.

Refolding was started by dilution of the sample with Tris-sulfate buffer to give a lower denaturant concentration. In the kinetic experiments, the concentration of I was varied from 15 to 120 μM. Usually, the other reaction conditions were: 20 μM enzyme, 1.0 M Gdn·HCl, 0.1 M Tris-sulfate buffer, pH 8.4, 25 °C. During refolding, continuous absorbance measurements were made at 545 or 485 nm relative to a reference lacking the enzyme but the same in other respects. At the end of the kinetic experiment, the reaction mixture and reference were stored in the dark, together with a control containing never denatured enzyme and I in the reaction buffer. Final absorbance measurements were generally made the following day; the absorbances of the reaction mixture and the control were not significantly different, showing that refolding was complete.

The kinetics of regeneration of the inhibitor site was also monitored by following the appearance of CD at 508 nm. This is a maximum in the extrinsic CD band generated by the binding of I to the enzyme (free I itself is optically inactive).

The equilibrium Gdn·HCl denaturation transition of the enzyme–I complex was followed by measuring the absorbance at 545 nm relative to a reference containing enzyme and I in 4 M Gdn·HCl. Each point in the transition was determined from an individual sample made up in a volumetric flask and measurements were repeated 24 h later. To check reversibility and ensure that equilibrium values were obtained, some points of the transition were determined by making up samples with enzyme that had first been denatured by exposure to >4.0 M Gdn·HCl.

Results

Equilibrium Transition. Bovine carbonic anhydrase–I complex is reversibly denatured by Gdn·HCl. At 545 nm the transition itself results in an absorbance decrease, while above and below the transition region the absorbance increases. The latter is evidently due to solvent perturbation of I and does not reflect enzyme conformational changes. Data were analyzed in terms of apparent fractional extent of denaturation, f_{ap}, given by

$$f_{ap} = (A - A_b)/(A_a - A_b) \quad (1)$$

where A is the absorbance at a given concentration of Gdn·HCl, and A_b and A_a represent the absorbance values before and after the transition, obtained by linear extrapolation. A plot of f_{ap} vs. Gdn·HCl is shown in Figure 1. The transition has a midpoint near 2.2 M Gdn·HCl and is virtually over by 4.0 M Gdn·HCl. Similar values of f_{ap} were obtained for the reverse renaturation.

The transition is broader and occurs at a higher Gdn·HCl concentration than reported for the enzyme alone (Yazgan and Henkens, 1972; Wong and Hamlin, 1975). This is not unexpected because preferential binding of I to the native state could stabilize it relative to others, and, in the same way, intermediate states that still have a I binding site could be stabilized relative to ones where the binding site is disrupted.

At 4.0 M Gdn·HCl, no binding of I is detected by either visible absorbance or CD measurements. At this concentration of denaturant, previous measurements have shown that the free enzyme is fully denatured (Yazgan and Henkens, 1972; Wong and Tanford, 1973), but the zinc ion is still coordinated (Stein and Henkens, unpublished).

Kinetics. During renaturation, sulfonamide I binds as soon as its binding site is regenerated and reports this by a change in its spectrum. Kinetic experiments that monitor changes in the absorbance of I are illustrated in Figure 2. The time course of the changes is described, within experimental uncertainty, by the sum of two exponential terms

$$\frac{A_\infty - A}{A_\infty} = P_1 e^{-\lambda_1 t} + P_2 e^{-\lambda_2 t} \quad (2)$$

where A and A_∞ are absorbances at time t and ∞ relative to a reference lacking the enzyme but the same as the sample in other respects, P_1 and P_2 are the relative amplitudes of the exponential terms, and λ_1 and λ_2 are macroscopic rate constants that characterize the time course of the change in A.

Figure 2 shows a plot of the logarithm of $(A_\infty - A)/A_\infty$ vs.

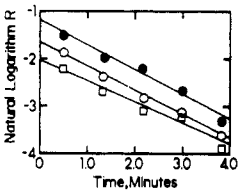

FIGURE 3: Rapid phase in the kinetics of change in the absorbance of I at 545 nm during enzyme refolding. Experimental conditions given in Figure 2. Kinetics described by first term of eq 2 in text, so that natural logarithm R = natural logarithm $P_1 - \lambda_1 t$.

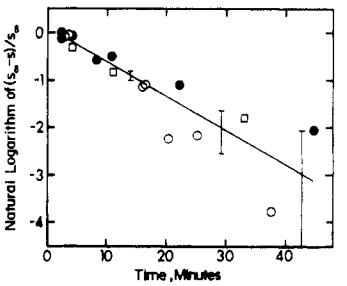

FIGURE 4: Kinetics of recovery of activity by the refolding enzyme. Refolding reaction conditions: 20 μM enzyme, 1.0 M Gdn·HCl, 0.05 M Tris-sulfate buffer, pH 7.8, 25 °C. Aliquots were removed from the reaction mixture and assayed for p-nitrophenyl acetate activity according to Yazgan and Henkens (1972); s and s_∞ equal to activity at time t and ∞, respectively. The error bars show the uncertainty due to the propagation of a ±4% uncertainty in activity. Different symbols indicate separate kinetic runs.

t. At large t, when the term containing the larger macroscopic rate constant, λ_1, becomes negligible, the semilog plot becomes linear with slope λ_2 and intercept log P_2. To obtain λ_1, $P_2e^{-\lambda_2 t}$ is substracted from $(A_\infty - A)/A_\infty$, and the log of the difference plotted against time. The slope of this plot gives λ_1, and its intercept gives log P_1. This analysis is illustrated in Figure 3.

Similar results, not shown, were obtained when CD was used to follow the reaction. Here, extrinsic visible CD arising from bound I is monitored. Such CD is not observed until I is bound to the enzyme.

Results for all the experiments, treated in the manner outlined, are given in Table I. Within experimental uncertainty, the values of λ_1 and λ_2 are virtually independent of enzyme, zinc(II), and inhibitor concentrations. In particular, variation of the concentration of I over approximately a tenfold range, from 15 to 120 μM, does not significantly affect λ_1 or λ_2, showing that binding of I is not rate limiting. Also, variation of the concentration of I does not change P_1 or P_2, suggesting stoichiometric binding of I.

This kinetic behavior is described by Scheme I, where the A_i's represent various conformational states of the protein, and I represents the inhibitor I. Steps 1, 2, and 4 represent enzyme conformational changes, the latter with I bound to the enzyme. The binding reaction (step 3) is taken to be essentially irreversible because variation of I does not affect P_1 or P_2. The last step (step 4) is written as irreversible because refolding is carried out at low Gdn·HCl concentrations, below the denaturation transition, where at equilibrium only the native state is present in significant amounts. A_1 does not necessarily represent the completely unfolded enzyme. As a matter of fact, far ultraviolet CD measurements that are given later in this report show that A_1 contains significant amounts of secondary structure and is rapidly formed from the fully unfolded molecule.

SCHEME I

$$A_1 \underset{2}{\overset{1}{\rightleftarrows}} A_2 \qquad \text{(slow)}$$

$$I + A_2 \xrightarrow{3} IA_2 \qquad \text{(fast)}$$

$$IA_2 \xrightarrow{4} IA_3 \qquad \text{(slow)}$$

Assuming only that inhibitor binding is fast compared with the conformational changes represented by steps 1, 2, and 4 (ie., $k_3[I] \gg k_1, k_2, k_4$), the differential equations describing the rate of change of concentrations in Scheme I can be solved (cf. Szabo', 1969), and in terms of Scheme I: $\lambda_1 = k_1$, $\lambda_2 = k_4$. Because $\lambda_1 \gg \lambda_2$, P_1 is equal to the relative optical change associated with the binding of I, and P_2 is the relative optical change occurring during the subsequent conformational change $IA_2 \rightarrow IA_3$.

It should be pointed out that, although the sum $P_1 + P_2$ seems to be constant (Table I), the average value from absorbance measurements is $P_1 + P_2 = 0.78$ (± 0.03 standard error) and the average value from CD measurements is $P_1 + P_2 = 0.91$ (± 0.03 standard error). Because of the possibility of systematic errors in the determinations of initial and final values of absorbance and CD, we are uncertain of the reliability of these values, especially the one determined from absorbance measurements. Further studies on this point are necessary. None-the-less, in both cases it appears that Scheme I does not fully account for the observed optical changes, for then $P_1 + P_2$ would be unity.

This could mean that a very fast refolding reaction is measured by I binding. In this case, another exponential term should be included in eq 2. And in terms of the reaction scheme, it would mean that either two intermediates are capable of binding I, or that both slow and fast reactions give rise to the same intermediate, A_2. The latter is more likely because of the constancy of $P_1 + P_2$. Analogous results were obtained with thermally unfolded ribonuclease A in that both the fast and slow refolding reactions give native enzyme (Garel and Baldwin, 1973). With bovine carbonic anhydrase, however, they appear to give only a quasi-native enzyme, and further very slow folding is required to give enzymatically active enzyme. The latter is clearly seen in the following studies on the recovery of activity.

Recovery of Activity. Additional information on the nature of the step $A_2 \rightarrow A_3$ is obtained from experiments on the kinetics of recovery of esterase activity. After a short induction period, apparent first-order kinetics is followed in the recovery of activity by the refolding enzyme (Figure 4). Within experimental error, the specific rate is the same as the slowest phase in changes in absorbance at 292 nm of the enzyme alone (Yazgan and Henkens, 1972) and the conformational changes in the enzyme-inhibitor complex. This shows that the presence of I does not detectably alter the rate, and that the conformational change $A_2 \rightarrow A_3$ involves folding of the protein in the active site region that both perturbs the spectrum of bound I and provides the necessary arrangement of atoms for enzymatic activity.

To confirm this result and further probe the dynamics of formation of the active site, we have developed a continuous assay method (p-nitrophenyl acetate as substrate) to monitor refolding. To begin the refolding reaction, a 10–50-μ aliquot of the fully denatured enzyme in >3 M Gdn·HCl is diluted with buffer (0.05 M Tris-sulfate (pH 7.0)) containing 1.0 mM p-nitrophenyl acetate. During the course of the refolding reaction, the absorbance at 400 nm (the spectral peak for p-

TABLE I: Kinetics of Refolding of Gdn·HCl Denatured Bovine Carbonic Anhydrase.[a]

Gdn·HCl (M)	[Probe][b] (× 10^5 M)	[Enzyme] (× 10^5 M)	$[Zn(II)]_{total}$ (× 10^5 M)	Method	$\lambda_1 \times 10^2$ (s^{-1})	$\lambda_2 \times 10^2$ (s^{-1})	P_1	P_2
0.52	1.5	1.3	1.3	CD[c]	2.4	0.13	0.21	0.70
0.94	2.0	1.6	1.6	CD	0.7	0.09	0.30	0.40
0.94	2.0	1.6	1.6	Abs[d]	2.3	0.18	0.25	0.50
0.96	2.0	1.6	1.6	CD	0.9	0.18	0.38	0.51
0.96	2.5	2.1	2.1	CD	1.3	0.11	0.39	0.53
0.96	3.0	1.9	3.5	Abs	1.6	0.12	0.20	0.58
0.96	3.0	1.9	6.7	Abs	I[f]	0.10	I[f]	0.70
0.96	6.0	2.3	5.0	Abs	I	0.11	I	0.57
0.96	6.0	1.9	1.9	Abs	2.4	0.19	0.19	0.58
0.96	9.0	2.1	2.1	Abs	1.0	0.15	0.32	0.57
0.96	12.0	2.0	2.0	Abs	1.5	0.17	0.17	0.32
1.00	2.0	1.7	1.7	Abs	2.4	0.18	0.22	0.73
1.00	3.0	1.9	1.9	Abs	0.9[e]	0.08[e]	0.21[e]	0.56[e]
1.00	3.0	1.7	1.7	Abs	2.4	0.19	0.24	0.57
1.00	3.0	2.0	2.0	Abs	I	0.14	I	0.70
1.00	3.0	3.4	3.4	Abs	I	0.16	I	0.64
1.00	3.0	6.9	6.9	Abs	1.9	0.16	0.23	0.66
1.00	3.5	2.6	2.6	Abs	I	0.18	I	0.47
1.00	4.0	1.7	1.7	Abs	1.8	0.23	0.20	0.44
1.00	6.0	2.0	2.0	Abs	1.2	0.16	0.32	0.51
1.00	6.0	2.1	2.1	Abs	2.3	0.16	0.24	0.66
1.11	2.0	1.6	1.6	CD	0.9	0.14	0.24	0.69
1.24	2.0	1.6	1.6	CD	0.9	0.17	0.35	0.63
1.24	2.0	1.6	1.6	CD	0.7	0.16	0.26	0.60
1.24	6.0	2.3	2.3	Abs	0.8	0.15	0.27	0.50
1.45	2.0	1.6	1.6	CD	0.4	0.10	0.13	0.75
1.50	3.0	2.0	2.0	Abs	I	0.08	I	0.78
1.50	6.0	2.0	2.0	Abs	0.7	0.08	0.12	0.64
1.50	9.0	2.0	2.0	Abs	1.8	0.09	0.17	0.53
1.55	2.0	1.6	1.6	CD	1.1	0.10	0.23	0.79
1.68	2.0	1.6	1.6	CD	1.1	0.06	0.12	0.91
1.92	2.0	1.6	1.6	CD	I	0.021	I	1.0
1.92	4.0	2.0	2.0	Abs	I	0.031	I	0.87
1.92	5.0	4.0	4.0	Abs	I	0.033	I	0.91

[a] Unless otherwise indicated, experiments carried out in 0.1 M Tris–sulfate buffer pH 8.4, 25 °C. Kinetic data analyzed according to method described in text. [b] Probe is compound I. [c] Kinetics followed by changes in CD at 508 nm. [d] Kinetics followed by changes in absorbance at 545 or 485 nm. [e] pH 6.0. [f] Indefinite values.

nitrophenolate ion) or at 347 nm (isosbestic point of the ionized and unionized forms of *p*-nitrophenol) is monitored. A representative experiment is illustrated in Figure 5.

The data are fitted to first order plots, but the analysis is somewhat different than usual because it is the time derivative of the absorbance, not the absorbance itself, that is proportional to the concentration of reactivated enzyme. Assuming a first-order reaction for the recovery of activity with specific rate k

$$[E] = [E]_\infty(1 - e^{-kt}) \tag{3}$$

where [E] is the concentration of reactivated enzyme at time t, and $[E]_\infty$ is the final concentration of reactivated enzyme. Assays are carried out under conditions where the concentration of substrate is approximately constant during the course of analysis, so that dA/dt is proportional to [E]. From this, it is easily seen that

$$dA/dt = s_\infty(1 - e^{-kt}) \tag{4}$$

where s_∞ is the limiting slope at large t of the plot of absorbance vs. time (Figure 5). Integration, assuming $A = 0$ at $t = 0$, gives

$$A = s_\infty t - [s_\infty(1 - e^{-kt})]/k \tag{5}$$

At large t, when reactivation is complete, the exponential term becomes zero, so that

$$A_r = [t - (1/k)] \tag{6}$$

Subtracting eq 6 from eq 5 and taking the natural logarithm gives

$$\ln(A - A_r) = [\ln(s_\infty/k)] - kt \tag{7}$$

Equation 7 shows that k can be evaluated from the slope of a plot of $\ln(A - A_r)$ vs. t. A representative plot is shown in Figure 5. It is important to mention, however, that a very short induction period in reactivation would not be detected in this analysis. Another complication is that the activity of the regenerated and the native enzyme is reduced by Gdn·HCl concentrations far below those that cause denaturation. This is quantitatively explainable by inhibition by chloride ion (Yeagle, 1974), a well known carbonic anhydrase inhibitor, and does not alter the kinetics.

The apparent first-order rate constants, k, were evaluated by linear least-squares analysis of plots like those shown in Figure 5, and are given in Figure 6. Over a substantial range, the kinetics of reactivation is virtually independent of the concentration of Gdn·HCl present during refolding, and also virtually independent of protein concentration (cf. results for

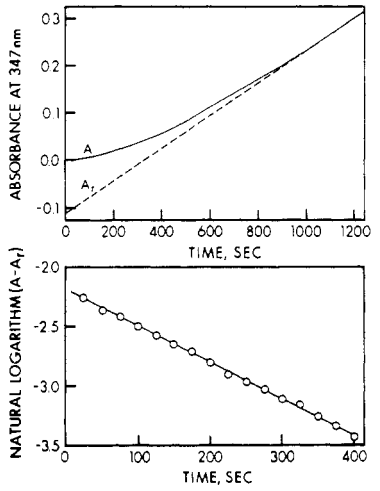

FIGURE 5: Recovery of enzymatic activity during refolding. When enzyme refolds in the presence of substrate (*p*-nitrophenyl acetate), there is a lag of several minutes before substrate hydrolysis, monitored by absorbance changes at 347 nm, proceeds at maximum rate. This phenomenon, which is illustrated in curve A, gives a measure of the rate of recovery of enzymatic activity. Utilization of the information to determine the rate constant for reactivation, through the means of a plot of logarithm $(A - A_f)$ vs. time, is described in the text.

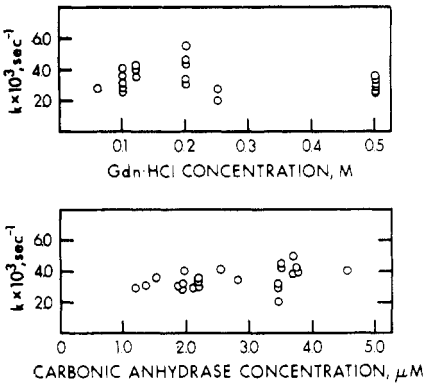

FIGURE 6: Rate constants, *k*, for the recovery of enzymatic activity during refolding. Values of *k* determined by a continuous assay method, which is illustrated in Figure 5 and fully described in the text.

TABLE II: Effect of Added Zinc(II) Ion on the Specific Rate of Reactivation, *k*, in 0.1 M Gdn·HCl, Variable Amounts of Enzyme, pH 7.0, 25 °C.[a]

Added Zinc(II) (equiv)	$k \times 10^2$ (s^{-1})
0.0	0.33
0.5	0.37
1.0	0.29
2.0	0.32
3.0	0.56
4.0	0.26

[a] The result for the case without added zinc ion is the average of ten experiments in which the enzyme concentration was between 1 and 3 μM; the result at 0.5 equiv of added zinc ion is the average of four experiments at 1 to 3 μM enzyme; the other results are from single experiments at 1 μM enzyme.

the human B enzyme (Carlsson et al., 1975)). Finally, as shown in Table II, reactivation kinetics is not measurably influenced by added zinc ion.

Changes in Optical Activity. The kinetics of refolding was also followed by continuously monitoring changes in circular dichroism at 220 and 230 nm. The measurements show that the optical activity increases very rapidly in this region of the spectrum during refolding. The major changes are completed before the first measurements, which take about 20 s. These are followed by small decreases in optical activity that occur over a period of several minutes. It is assumed that the major changes in optical activity in this region of the spectrum reflect backbone folding. This means that A_1 contains a substantial amount of secondary structure, and that, by the time A_2 has formed, the secondary structure as judged by the circular dichroism at 220 and 230 nm is similar to that of the final native state, which in carbonic anhydrase consists of a core of β structure and some helical segments. Further studies on backbone folding are in progress.

Discussion

From these studies it is evident that a quasi-native state capable of binding a specific inhibitor is formed at an intermediate stage in the folding process, prior to the recovery of the active conformation. Folding to the quasi-native intermediate forms enough of the active site crevice to provide a strong binding site for I. Binding appears stoichiometric at [I] = 20 μM, indicating that the stability constant is greater than 10^5 M^{-1}. Further folding to the active conformation is accompanied by comparatively large changes in absorption due to tryptophan side chains (Yazgan and Henkens, 1972) but is accompanied by only small changes in circular dichroism in the peptide backbone region of the spectrum.

On the basis of the results given here and elsewhere (Yazgan and Henkens, 1972), we can identify three intermediates in the pathway of folding from the randomly coiled polymer to the native structure. They are: (1) a highly disorganized polypeptide (**1**) containing bound Zn^{2+}, likely coordinated to His-93 and His-95, and possibly other groups as well; (2) an intermediate (**2**) containing a substantial amount of secondary structure; and (3) a quasi-native structure (**3**) capable of binding the specific inhibitor I. Combining the probe, activity, and direct spectroscopic observations, three time constants are detected. These are: less than 20 s, about 1 min, and about 10 min. These are tentatively assigned to **1** → **2**, **2** → **3**, and **3** → native structure.

It is commonly thought that, although partial protein folding might be observable by physical means, substrate binding and catalytic activity are likely to require complete folding. For bovine carbonic anhydrase, recovery of esterase activity does seem to require complete folding, but these probe experiments show that a tight binding site for a specific inhibitor is regenerated at an earlier stage.

To determine the dynamics of formation of a specific site, the only requirement is that probe binding must be fast compared with protein folding. Of course, subsequent folding in the region may be modified by the presence of the bound probe, but this seems to be small with bovine carbonic anhydrase. Similar methods could be used with other enzymes to study the kinetics and mechanism of regeneration of specific sites—for example, the active site.

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Near-Infrared Magnetic Circular Dichroism of Cytochrome *c'* †

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ABSTRACT: The near-infrared magnetic circular dichroism (MCD) of *Rhodospirillum rubrum, Chromatium vinosum,* and *Rhodopseudomonas palustris* cytochromes *c'* are reported. The spectra of the reduced protein are very similar to those of deoxymyoglobin. The spectra of the oxidized proteins in the pD range 1–13 can be analyzed on the basis of four species A, B, C, and D. The existence of nine species, reported in a recent electron paramagnetic resonance study, is not substantiated. The MCD spectra support the assignment of B as high spin and C and D as low spin. The MCD of species A is close to that of high-spin proteins and does not support the recently proposed assignment of a mixed high- and intermediate-spin ground state for this species. The energies of the near-IR electronic transitions of all four oxidized species point to axial ligation via oxygen, assuming histidine to be the opposite axial ligand. Unfortunately, insufficient model compounds with ligation by carboxyl or hydroxyl moieties exist to enable more precise assignments.

The cytochromes *c'* are atypical heme proteins (for recent reviews, see Kamen and Horio, 1970; Kamen et al., 1972). In the reduced state, their optical spectra and magnetic susceptibility are myoglobin-like, but the ligand binding characteristics are substantially different (Taniguchi and Kamen, 1963). In the oxidized state at neutral pH, their optical spectra, magnetic susceptibility, and EPR[1] most resemble high-spin ($S = 5/2$) acid metmyoglobin. However, the room-temperature magnetic moment is below the usual high-spin range and the low-temperature EPR differs from that expected (Ehrenberg and Kamen, 1965). The resonance Raman (Strekas and Spiro, 1974) and Mössbauer (Moss et al., 1968) spectra are also unusual. In addition, the anion binding behavior is unlike that of acid metmyoglobin. The magnetic properties were originally interpreted in terms of a spin-state equilibrium (Ehrenberg and Kamen, 1965). However, very recently it has been proposed (Maltempo et al., 1974; Maltempo, 1974; Maltempo and Moss, 1976) that the ground state consists instead of a novel admixture of high- and intermediate-spin ($S = 5/2$ and $3/2$) components not encountered previously. Oxidized cytochromes *c'* also exhibit complex behavior with pH variation (Taniguchi and Kamen, 1963; Horio and Kamen, 1961). In a recent detailed EPR study (Maltempo et al., 1974; Maltempo, 1974, 1975; Maltempo and Moss, 1976), nine magnetically distinct species have been reported in the pH range 1–13.

We present here measurements of the magnetic circular dichroism (MCD) (Stephens, 1974, 1976) of cytochrome *c'* in the near-infrared spectral region. The near-IR electronic transitions of heme proteins and their derivatives are more sensitive to the ligation, oxidation state, and spin state of the heme iron than are the visible (α, β, and Soret) transitions (Smith and Williams, 1970). MCD both enables these transitions to be more easily detected and yields more information than absorption spectroscopy (Cheng et al., 1973; Stephens et al., 1976). Thus, for example, the MCD of the near-infrared transitions of high- and low-spin ferric hemoglobin derivatives are diagnostic of the ground-state spin state (Stephens et al., 1976). Our objective has been to examine the information content of near-IR MCD in the case of the various forms of cytochrome *c'*. Specifically, we have studied the *c'* cytochromes obtained from the photosynthetic bacteria *Rhodospirillum*

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; IR, infrared; MCD, magnetic circular dichroism.