B. N. Moiseyev, L. V. Popov, *Izv. Acad. Sci. USSR Phys. Solid Earth* **1**, 13 (1970).
19. L. Liu, *Nature (London)* **262**, 770 (1976).
20. ———, *Earth Planet. Sci. Lett.* **31**, 200 (1976); *Science* **195**, 990 (1977).
21. ———, *Geophys. J. R. Astron. Soc.* **48**, 53 (1977); *Earth Planet. Sci. Lett.* **36**, 237 (1977).
22. I thank J. R. Cleary for commenting on the manuscript. This research was supported by the California Institute of Technology's President Venture Fund. Contribution No. 2941, Division of Geological and Planetary Sciences, California Institute of Technology.
* On leave of absence from the Research School of Earth Sciences, Australian National University, Canberra, A.C.T., Australia.

18 July 1977; revised 12 September 1977

Far-Ultraviolet Stopped-Flow Circular Dichroism

Abstract. *A stopped-flow circular dichroism instrument, with a total accessible wavelength range of 200 to 750 nanometers, has been constructed and provides a spectroscopic method for kinetic investigations of a wide array of fast reactions in which optical activity changes in absorbing regions are involved. An important biochemical application depends on the far-ultraviolet capability, which allows observation of the rapid alterations in backbone conformation associated with folding and unfolding reactions of proteins. Results obtained by following two such reactions at 222 nanometers represent direct monitoring by circular dichroism of rapid secondary structure changes in proteins.*

The elucidation of the detailed processes involved in biological macromolecular folding represents a fundamentally important area of current biochemical research (*1*, *2*). As part of an effort in that direction, we have been investigating thermodynamic and kinetic aspects of the folding from partially disordered states of separated, human α- and β-globin chains free of heme (α^0 and β^0). Early experiments (*3*, *4*) revealed the need for instrumentation which would provide a direct and interpretable probe of rapid secondary structure changes. Toward that end, we have constructed a prototype stopped-flow circular dichroism (SFCD) instrument with a time response in the millisecond range in the far-ultraviolet (UV) absorption region of the protein polypeptide backbone.

In contrast to light transmission and fluorescence—the parameters most often monitored in stopped-flow (SF) work—circular dichroism (CD), which measures differential absorbance of left- and right-circularly polarized light (*5*), is intrinsically sensitive to molecular asymmetry. The SFCD technique in the visible and near-UV was introduced by Bayley and Anson (*6*) with measurements of the changes in ligand-induced CD at selected wavelengths for several protein systems. More recently, Nitta *et al.* (*7*) have reported the application of SFCD for following changes in ellipticity at 270 nm of aromatic amino acid residues.

The CD associated with spectral transitions near 220 nm of the polypeptide backbone provides a direct measure, for a protein in solution, of the type and the extent of secondary structure (*8*). By use of a stabilized Xe light source, a 50-khz piezooptical birefringence modulator, and phase-sensitive, heterodyning lock-in amplification techniques, in conjunction with an observation chamber of novel optical and flow design (*9*) and a carefully constructed vibration-damping mount, we have overcome the considerable light energy and stability problems involved in making rapid far-UV measurements with acceptable signal-to-noise (S/N) ratios. Modifications are being made to improve signal sensitivity and time response by an order of magnitude. Details of the instrumental designs may be found elsewhere (*10*).

As examples of the application of the prototype instrument to monitor far-UV CD changes, we present here two experiments of importance to our hemoglobin studies. In the first we monitored partial refolding when a solution of disordered α^0 was brought from *p*H 4.5 to *p*H 5.8. In the second we measured the rapid acid denaturation of methemoglobin by following loss of α-helix as seen in the fall of CD intensity at 222 nm. It should be noted that in both cases the parameter being followed provided a measure of the rate of change in α-helix content. To our knowledge, the results represent the first direct detection of rapid secondary structure changes and allow isolation of one level of conformational change in complex systems where many occur.

The band pass at 222 nm was ~2.5 nm. Instrumental dead time, the time elapsed between the initiation of mixing and the beginning of accumulation of useful kinetic data, was 12 msec. The data, representing the difference in transmission between the 50-khz alternating left- and right-circularly polarized components—a value approximately 10^{-4} times the magnitude of the average light transmission—as normalized with respect to the average, were digitally recorded and displayed. To avoid distorting effects, the lock-in amplifier output smoothing time constant, τ_F, was always set at less than 0.1 times the value of the observed reaction lifetime. The photographs of the displayed data (see Figs. 1 and 2) show the raw data points from consecutive SF runs with no adjustment of instrumental setting or averaging. Each SF run involved an expenditure of 0.15 ml of reactant.

The α^0 was prepared and purified at *p*H 5.7 (20 m*M* phosphate) by the method of Yip and co-workers (*3*, *4*). The isolated monomer fraction was denatured by adjusting the *p*H to 4.5 with 1*M* KH_2PO_4. The jump to *p*H 5.8, carried out at 4°C, was accomplished by mixing with 20 m*M* K_2HPO_4 (see Fig. 1 and Table 1). The rise in the CD value of the *p*H-jumped globin represents an increase in the percentage of amino acid residues in the helical conformation (percent helicity)

Table 1. Stopped-flow 222-nm circular dichroism experiments. The concentrations of the initial protein reactant solutions were determined by absorbance measurements, using extinction coefficients for methemoglobin of $\epsilon_{500} = 9.04 \times 10^3$ and $\epsilon_{406} = 162 \times 10^3$ per heme (*13*) and for α^0 of $\epsilon_{277.5} = 10 \times 10^3$ (*3*). Static CD values were determined on a Cary recording spectropolarimeter in the static mode, using a model 6001 CD accessory. The actual SF dead time was 12 msec, but the apparent dead time depends on τ_F.

Experimental parameter	Renaturation of α^0	Denaturation of methemoglobin
*p*H of protein reactant solution	4.5	6.5
Final protein concentration, mole/liter	2.2×10^{-5}	1.8×10^{-5} (in heme)
Total CD signal		
Degrees	0.023	0.088
Degree cm^2 dmole^{-1} (mean residue)	3.7×10^3	17.2×10^3
Associated noise level, millidegrees	~5	~17
Second reactant	20 m*M* K_2HPO_4	HCl
*p*H of second reactant	9.5	1.31, 1.76*, 2.26
Final *p*H of mixture	5.8	1.55, 2.05, 2.55
Total time span of photograph, seconds	102.4	1.02, 1.02, 5.1
$\tau_{1/2}$, milliseconds		
Approximate from photograph		48, 110, 455
From digital analysis	5000	42, 124, 415

*Photograph not shown.

0036-8075/78/0127-0425$00.50/0

of ~10 (*11*); the full 222-nm CD value of α^0 monomer at *p*H 5.7 was recovered.

For the acid denaturation unfolding experiments, unbuffered human methemoglobin was prepared from centrifuged hemolyzates by reaction with potassium ferricyanide. Excess reagent was separated by chromotography on a Bio-Gel P-2 column and the eluted protein was reacted, at 20°C, with freshly prepared HCl solutions. For the two denaturations shown in Fig. 2, the parameters of interest are given in Table 1. An approximate order of magnitude drop in the half-lifetime ($\tau_{1/2}$) of α-helix loss for a decrease of one *p*H unit is apparent from a comparison of Fig. 2, a and b. Digital analysis of the experimental results gives $\tau_{1/2} = 1.15 \times 10^3$ mole liter^{-1} sec $[H^+]^{-1}$ (*12*) over the *p*H range covered. The total drop in 222-nm intensity represents a decrease in percent helicity of ~60 (*11*). The associated noise level is typical at 222 nm for samples with an effective absorbance of 0.5 to 1.5 units when a τ_F of 4 msec is used. The noise level increases and hence the S/N degrades slowly beyond this optimal absorbance range. The noise level also varies approximately as $\tau_F^{-1/2}$. The S/N improves sharply with increasing wavelength, yielding (for optimal absorbance at each of the wavelengths cited) a threefold improvement over the 222-nm value at 227 nm, a sixfold improvement at 260 nm, and a ninefold improvement at 290 nm.

As demonstrated by these results, the present instrument is capable of following a variety of processes involving secondary structure changes. The modified instrument will, in addition to providing for more sensitive SF monitoring of CD, readily allow for monitoring of SF transmission, fluorescence, and light scattering. These various spectroscopic monitoring techniques, throughout the accessible wavelength range of 200 to 750 nm, provide information about changes on different structural levels. This is particularly important in complex systems such as those involving both aggregation and refolding (for instance, $\alpha^0 + \beta^0 \rightarrow \alpha^0\beta^0$, with the dimeric apohemoglobin product being more thoroughly folded than either of the monomeric reactants). Thus, while the SF light transmission change at 222 nm associated with the simple system of the α^0 monomer *p*H jump (~10^{-4} absorbance units) exhibits the same basic time behavior as the CD results ($\tau_{1/2} = 5$ seconds for both changes under the conditions of Table 1), for the more complex protein reaction cited above, the CD shows a time dependence different from that of the other monitoring signals. Thus with each reactant at a concentration of $1.1 \times 10^{-5}M$, at *p*H 5.7, $\tau_{1/2}$ for transmission at 222 nm is 17 seconds, while $\tau_{1/2}$ for CD is 130 seconds (*10*).

The instrument described here adds to our ability to separate the various levels of structural information which are critical to a comprehensive understanding of the processes involved. We have emphasized its far-UV CD capabilities because of their application to protein folding and assembly kinetics. However, the sharp enhancement in instrument performance at longer wavelengths points to the value of SFCD not only in biochemical studies but also in diverse kinetic studies, in physical, organic, and inorganic chemistry, where altered optical activity and modification of chiral centers are involved.

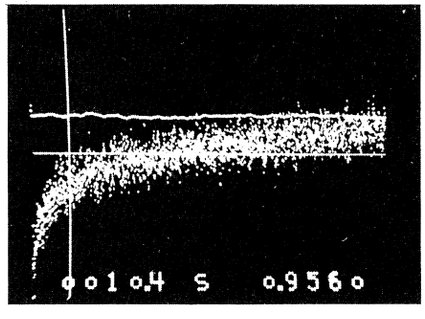

Fig. 1. Renaturation by jumping the *p*H of the solution, monitored at 222 nm by SFCD. A *p*H 4.5 solution of $4.4 \times 10^{-5}M$ α^0 monomer was prepared by mixing five volumes of $5.3 \times 10^{-5}M$ α^0 monomer at *p*H 5.7 and 20 m*M* phosphate with one volume of 1*M* KH_2PO_4. The *p*H was jumped to 5.8 by SF mixing with an equal volume of 20 m*M* K_2HPO_4. The upper horizontal trace is the final (*p*H 5.8) CD value; the lower horizontal line is an electronic screen center marker. The multiple point at the bottom left is within 5 percent of the expected dilution CD value (for $2.2 \times 10^{-5}M$ α^0 monomer at *p*H 4.5; the dilution value being that expected from a one-to-one dilution of protein solution, with no secondary structure change). The data are for three consecutive SF runs superimposed, with no averaging. The τ_F for the runs was 40 msec. (The position of the vertical line and the value of the corresponding numeric are arbitrary.) Total time span, 102.4 seconds; final CD value obtained with a τ_F of 1.25 seconds; sampling rate, 100 msec per point.

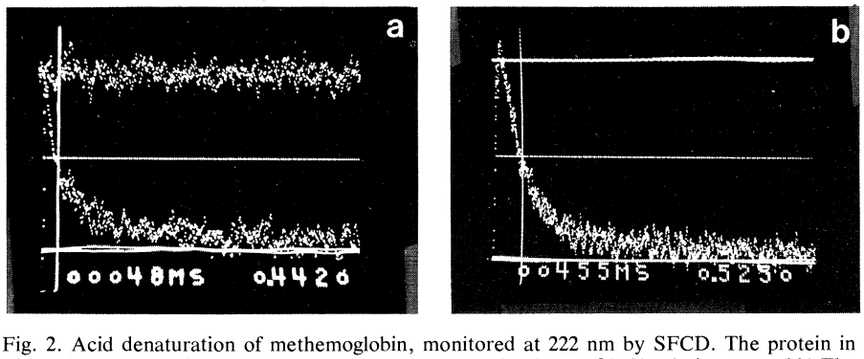

Fig. 2. Acid denaturation of methemoglobin, monitored at 222 nm by SFCD. The protein in water, $3.6 \times 10^{-5}M$ in heme, was reacted with an equal volume of HCl solution. (a and b) The upper horizontal trace is the dilution CD value; the middle one, an electronic screen center marker; and the lower one, the final CD value of the denatured methemoglobin. In each photograph, two SF runs are shown superimposed, with no averaging. For those runs, τ_F was 4 msec. Approximate half-reaction is indicated by the position of the vertical marker; the corresponding $\tau_{1/2}$ value is given by the left-hand numeric display. (a) The *p*H of the HCl solution was 1.3; the final *p*H of the mixture, 1.6; total time span of photograph, 1.02 seconds; for the dilution value trace, $\tau_F = 4$ msec; final value trace, $\tau_F = 0.4$ second; sampling rate, 1 msec per point. (b) The *p*H of the HCl solution was 2.3; the final *p*H of the mixture, 2.6; total time span, 5.1 seconds; for both dilution and final values, $\tau_F = 0.4$ second; sampling rate, 5 msec per point.

J. Luchins, S. Beychok
Departments of Chemistry and *Biological Sciences, Columbia University, New York 10027*

References and Notes

1. R. L. Baldwin, *Annu. Rev. Biochem.* **44**, 453 (1975).
2. F. M. Richards, *Annu. Rev. Biophys. Bioeng.* **6**, 151 (1977).
3. Y. K. Yip, M. Waks, S. Beychok, *J. Biol. Chem.* **247**, 7237 (1972); M. Waks, Y. K. Yip, S. Beychok, *ibid.* **248**, 6462 (1973); Y. K. Yip, M. Waks, S. Beychok, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 64 (1977).
4. S. Beychok, J. Luchins, M. Waks, Y. K. Yip, *Abstr. Pap. 172d Natl. Meet. ACS* (1976), abstr. 2; Y. K. Yip, J. Luchins, S. Beychok, in preparation.
5. L. Velluz, M. Legrand, M. Grosjean, *Optical Circular Dichroism* (Academic Press, New York, 1965).
6. P. M. Bayley and M. Anson, *Biopolymers* **13**, 401 (1974); *J. Phys. E* 7, 481 (1974); *Biochem. Biophys. Res. Commun.* **62**, 717 (1975).
7. K. Nitta, T. Segawa, K. Kuwajima, S. Sugori, *Biopolymers* **16**, 703 (1977).
8. S. Beychok, *Science* **154**, 1288 (1966).
9. We are indebted to Q. Gibson for suggestions about the design of the observation chamber.
10. J. Luchins, thesis, Columbia University (1977).
11. See, for example, Y. H. Chen and J. T. Yang [*Biochem. Biophys. Res. Commun.* **44**, 1285 (1971)] for methods of estimating α-helix content.
12. H. Polet and J. Steinhardt, *Biochemistry* **8**, 857 (1969); W. Allis and J. Steinhardt, *ibid.*, p. 5075.
13. O. Van Assendelft, *Spectrophotometry of Haemoglobin Derivatives* (Royal Vangorcum, Ltd., Assen, Netherlands, 1970).
14. This work was supported in part by NSF grant PCM 77-08537.

30 September 1977; revised 14 November 1977