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STOPPED-FLOW CIRCULAR DICHROISM AS A DIRECT PROBE OF RAPID CONFORMATIONAL CHANGE OF A PROTEIN. REDUCTION OF FERRICYTOCHROME C FROM HORSE HEART

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The elucidation of detailed processes involved in rapid macromolecular conformational changes of biological significance represents a fundamentally important area of recent biochemical research.[1] Among many biophysical techniques applied for the purpose, circular dichroism is intrinsically most sensitive to molecular asymmetry and, therefore, is a substantial probe of structure change.[2] We present here the first demonstration that stopped-flow circular dichroism spectroscopy (SFCD) not only is applicable to rapid transient kinetics but also provides a direct probe of rapid secondary and/or tertiary structure change of biological macromolecules. Now the authors wish to report the application of SFCD technique[3] to reduction of ferricytochrome c (Fe^{III}) by sodium dithionite in alkaline solution at various wavelengths. The recorded rapid change of CD spectrum in a wavelength range of 385 to 430 nm for the first time clarified that at least two interpretable transient species were involved in the process.

A SFCD instrument (250 W Xe light, photoelastic birefringence modulator, and heterodying lock in amplification[4]) was modified to improve signal sensitivity. The instrument has a satisfactory time response (1/13 m sec), which was used in conjunction with an improved flow cell of better optical and flow design.[5]

Figure 1 shows the displayed data without further treatment after rapid mixing of an aqueous solution of 10 μM ferricytochrome c from horse heart (Sigma.

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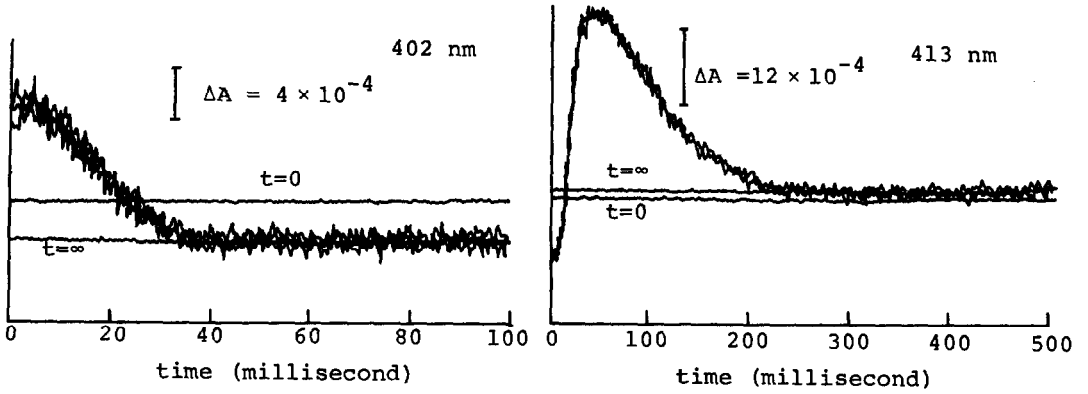
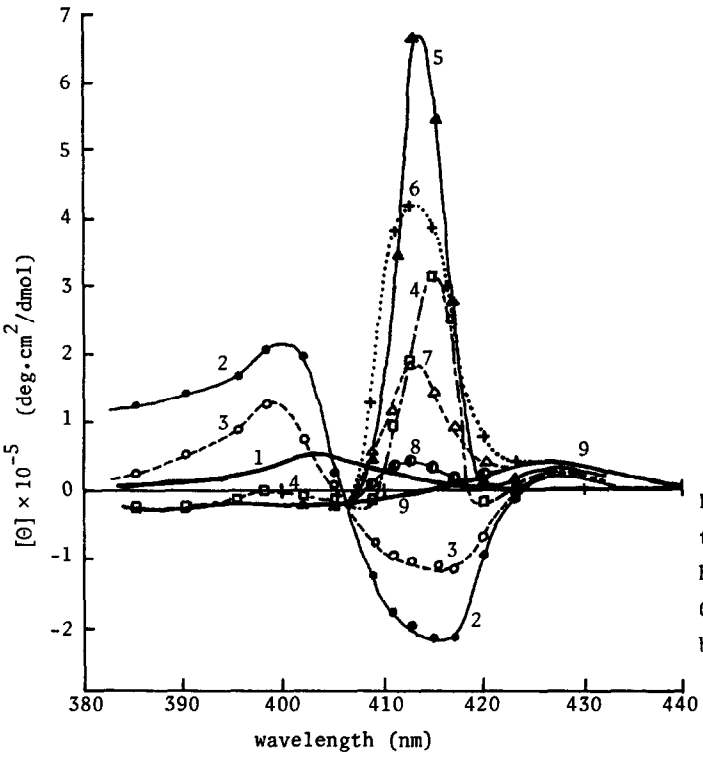


Figure 1. Stopped-flow circular dichroism of the reduction of ferricytochrome c from horse heart by sodium dithionite. Two or more independent traces are satisfactorily superimposed. Cyt c, 10μM; $Na_2S_2O_4$, 5.0 mM; pH 10.0; 25 mM tetraborate bubber; 28°. Response 55μsec. Traces at t = 0 (ferricytochrome c) and t = ∞ (ferrocytochrome c) are those treated by use of averaging system.

type III) with 5.0 mM sodium dithionite[7] in a 25 mM tetraborate buffer at pH 10. The decay of the 402 nm absorption[8] is essentially complete within 40 m sec (k_1, 200 sec^{-1}, 28°) and the rate is dependent on the dithionite concentration (0.2—5.0 mM).[9] However, the SFCD spectrum monitored at 413 nm strongly indicates rapid appearance of a transient species which is followed by a slower decay. It is evident from Figure 2 that the decay of the 402 nm band,[10] viz., the rapid electron transfer from dithionite to the heme c is synchronous with the rapid appearance of transient species having CD maximum at 413 nm. Thus, SFCD could clearly discriminate a transient (unstable) cytochrome c in the Fe^{II} form[11] from ferrocytochrome c (Fe^{II} form) having Met-80 as the sixth ligand. The Fe^{III} form of cytochrome c in alkaline solution[12] has Lys-79 as the sixth ligand in place of Met-80. Therefore, cytochrome c (Fe^{II} form) rapidly produced by the electron transfer either preserves the same secondary and tertiary structure as that of Fe^{III} form or still holds some oxidized form of "dithionite" as the sixth ligand. The electron transfer was followed by relatively slow relaxation of the unstable Fe^{II} form to the native Fe^{II} form, with the concomitant exchange of the sixth ligand of heme c to Met-80. The rate of slower decay of the transient species (17.3 ± 0.9 sec, 28°C) is independent on the dithionite concentration (0.2—5.0 mM). Therefore, the CD band at 413 nm is attributed to the transition of very



(1) ferricytochrome c, t=0
(2) 10 msec
(3) 20 msec
(4) 30 msec
(5) 40 msec
(6) 100 msec
(7) 150 msec
(8) 200 msec
(9) ferrocytochrome c, t=∞

Figure 2. Rapid CD spectrum in reduction of ferricytochrome c from horse heart by sodium dithionite.
Cyt c, 10 μM ; $Na_2S_2O_4$, 5 mM; tetraborate buffer, 25 mM; pH 10, 28°.

small electric moment and very large magnetic moment.

The rapidly and remarkably enhanced ellipticity in spectrum 2 at the initial stage compared with native ferricytochrome c is noteworthy and of considerable theoretical interest. An asymmetric position of a reductant with respect to heme c in the complex can reasonably account for the remarkably enhanced Soret rotational strength, since the origin of the magnetic dipole character of the Soret is assigned to the coupling with a conjugated electron system asymmetrically positioned near the heme.[13] This mechanism is also supported by our independent SFCD experiment on "dithionite reduction" of methemoglobin or metmyoglobin.[14]

From the present result it is concluded that SFCD is one of the most powerful techniques to detect rapid change of molecular environment in macromolecules of biological significance.

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4. Union CD-1002.
5. A flow cell was very carefully made from optical flat quartz and epoxy resin. The inner diameter of the flow channel is 4 mm in order to decrease the "dead time" in stopped flow technique.
6. Much faster than that in the SFCD techniques reported in Ref. 3 (< 1 m sec).
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9. The mechanism of so-called "dithionite reduction" is very complex; Ref. 7.
10. The transient species with λ_{max} (CD) at 400 nm and the large [θ] is discriminated from the native ferricytochrome c (spectrum 1, Fig. 2).
11. The electronic absorption maximum of the transient species is very close to that of ferrocytochrome c, strongly supporting Fe in ferrous valency state: Ref. 6b; I. Pecht and M. Faraggi, Proc. Nat. Acad. Sci. USA, 69, 902 (1972); E. J. Land and A. J. Swallow, Archives Biochem. Biophys., 145, 365 (1971).
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