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# Temporal Resolution of Individual Steps in an Enzymic Reaction at Low Temperature

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**Abstract.** Media are described which make it possible to study mechanisms of enzyme action at low temperature. The applicability of techniques developed are illustrated by results obtained on formation kinetics of complexes produced by the interaction of horseradish peroxidase with hydrogen peroxide. It is shown that the various steps in the time course of the reaction can be readily resolved with isolation of each intermediate in concentrations sufficient to permit rate studies between consecutive steps. The potential of the method for elaboration of enzyme mechanisms is discussed.

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Spectroscopic observation of transient intermediates in enzymic reactions might be made possible by manipulation of these systems in fluid media at low temperatures. Thus, the reactions should be considerably retarded and some intermediates accumulated to a detectable level. This procedure should permit one to obtain certain structural data, including those on conformational changes of enzyme molecules.

The main problem is to find appropriate solvents, that is, those which remain fluid at low temperature ($\ll 0°C$) and still dissolve native enzymes. Since 1964, it has been known that at least some enzymes can be dissolved and their reactions studied in aqueous alcoholic solvents (methanol-water) at low temperatures.[1,2] Since then, we have studied the variation of the physicochemical parameters of several of these mixtures from room temperature to their freezing points, i.e., density and viscosity,[3] dielectric constant,[4] and dissociation of weak electrolytes (acids, bases, buffers).[5,6]

Based on these data, we can control the polar character of such solvents at any given temperature or in a range of temperatures down to $-80°C$ and thus experiment with many proteins (in fact we have now tried up to 14 proteins) in the mixtures methanol-water (volume ratio: 70:30, 60:40, 50:50), ethylene glycol-water (50:50), dimethylformamide-water (70:30), dimethylsulfoxide-water (50:50).[7–10]

Recently, we have investigated under such conditions reactions catalyzed by horseradish peroxidase. The kinetic study of the transient appearance of intermediates in these systems was pioneered and more recently refined by Chance, who characterized the intermediates spectroscopically.[11]

By quenching the reaction at different temperatures we stabilized the intermediates, accumulated them to a recordable level, and finally interconverted

them by slight warming. In these conditions it was possible to study the intermediates both by spectrophotometry and by optical rotatory dispersion (ORD).

**Materials and Methods.** The horseradish peroxidase used was type VI (Sigma) with a Reinheitszahl value (absorption at 403/275 nm) of 3.0 and an activity of 295 purpurogallin units/mg. Hydrogen peroxide was a Merck product and luminol was obtained from Aldrich Chemical Company. Two hydro-organic mixtures were used: ethylene glycol-water in the volume ratio 50:50 (freezing point: $-55°C$) and dimethylformamide-water in the volume ratio 70:30 (freezing point: $-67°C$).

The first acts as a hydrogen donor toward the first enzyme-substrate complex (I) which is instantly transformed into Complex II. The results presented here were obtained with the dimethylformamide-water mixture between $-65°C$ and $+10°C$. The samples were prepared in the following manner: an aqueous solution of enzyme at 0°C (without freezing) was added to a small quantity of the organic solvent. The temperature was then lowered several degrees before further addition of solvent and the process repeated until the desired temperature was obtained.

The solutions were made in sodium phosphate buffer $2 \times 10^{-3}$ M (pH 8.0 in aqueous solution). It had been previously established that the phosphate buffer pH did not vary greatly with temperature (less than +0.2 unit between +20 and $-50°C$).[6]

Absorption and optical rotatory dispersion spectra were performed with a Cary 15 and a spectropolarimeter FICA respectively. Cells containing the samples were surrounded by double-walled metallic cryostats connected to a cooling-heating system. A controlled flow of gaseous nitrogen cooled by bubbling through liquid nitrogen circulated through the cryostats. The temperature of the cryostats was controlled by heating a resistor in a current of nitrogen ($\pm 0.2°C$) and the exact temperature of the sample was read on a galvanometer connected to a thermocouple placed inside the cell.[12] In these conditions, recording could be performed between $-100$ and $+80°C$, at any temperature.

The low temperature solutions were fluid and transparent. Their specific density and viscosity as a function of temperatures were measured.[3]

Substrates were mixed with the enzymic solution inside of the cryostat cells by means of constant magnetic stirring.

ORD spectra were recorded at the following enzyme concentrations: $1.17 \times 10^{-5}$ M for wavelengths $\lambda < 450$ nm and $6.7 \times 10^{-5}$ M for wavelengths $\lambda > 450$ nm. In both cases, complex intermediates were obtained at a concentration of hydrogen peroxide which was 1.5 times that of the enzyme.

**Results and Discussion. Absorption spectra of intermediates:** A buffered solution of dimethylformamide-water (volume ratio 70:30) containing equimolar amounts of horseradish peroxidase (final concentration 4.5 $\mu$M) was brought to $-65°C$ and the corresponding absorption spectrum recorded (Fig. 1). A stoichiometric quantity of hydrogen peroxide was then added and mixed with the solution at constant temperature. A new absorption spectrum appeared within 45 min and could be identified as the spectrum of the Complex I (Fig. 1).

By warming to $-20°C$, it was possible to record the appearance of a new spectrum (Fig. 1), which could be stabilized by sudden cooling to $-50$ or $-60°C$.

We see from Figure 1 that the three absorption spectra are similar to those previously obtained in aqueous solution by B. Chance using fast techniques.[11] The differences between both types of experiments lie in the fact that with fast techniques it is necessary to use a large excess of substrate to make the reaction and intermediates recordable, and that with the low temperature procedure such a recording can be performed with stoichiometric quantities of enzyme and substrate.

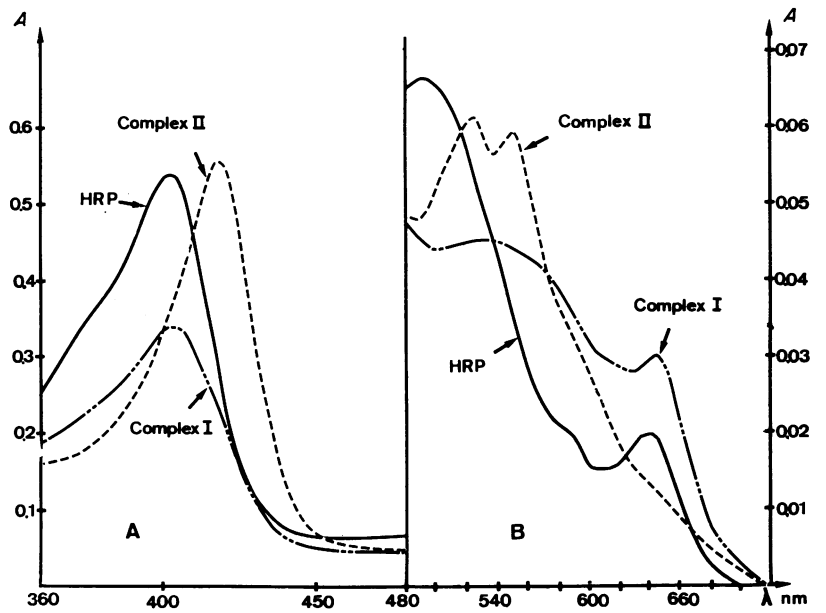


FIG. 1.—Absorption spectra of the horseradish peroxidase (HRP) and of *Complexes I* and *II* in the dimethylformamide-buffered water mixtures (70:30) at −65°C (fluid solvent). *HRP*, $H_2O_2$ and luminol concentrations were $4.45 \times 10^{-6}$ M. *A*, Soret band; *B*, visible spectra.

Kinetic studies were performed in the present studies on Complexes I and II: Complex I, stabilized at −65°C, could be transformed into Complex II, by heating to −50, −40, and −30°C in less than 1 min. Under these conditions, it was easy to follow the conversion between I and II as a function of time by measuring the increase in absorbance (*A*) at 428 nm (Fig. 2). The corresponding rate constants of this conversion were: $1.3 \times 10^2$ $M^{-1}$ $sec^{-1}$ (−50°C), $3.3 \times 10^2$ $M^{-1}$ $sec^{-1}$ (40°C), $11.1 \times 10^2$ $M^{-1}$ $sec^{-1}$ (30°C), compared with $2.8 \times 10^6$ $M^{-1}$ $sec^{-1}$ in aqueous solution at room temperature.

**Optical rotatory dispersion spectra:** It was determined that the ORD spectrum of the enzyme in the dimethyl formamide-water mixture (70:30) at −60°C was similar to that recorded in aqueous buffered solution at +10°C. An identical spectrum was obtained in the ethylene glycol-water mixture (50:50) at −40°C.

The addition of $H_2O_2$ to the enzymic solution (dimethylformamide-water at −60°C) resulted in the formation of Complex I for which the ORD spectrum was recorded (Fig. 3). Warming up to +10°C produced Complex II. Upon cooling, Complex II was stabilized and its ORD spectrum recorded at −60°C. In both cases, it was possible to check the presence of each complex by monitoring their approximate absorption spectra with the spectropolarimeter.

In Figure 3, it can be seen that the Cotton effect of the Complex I at 403 nm (Soret band) is different from that of the native enzyme and rather similar to that of the apoenzyme.[13] A hypochromic effect is observed in both cases.

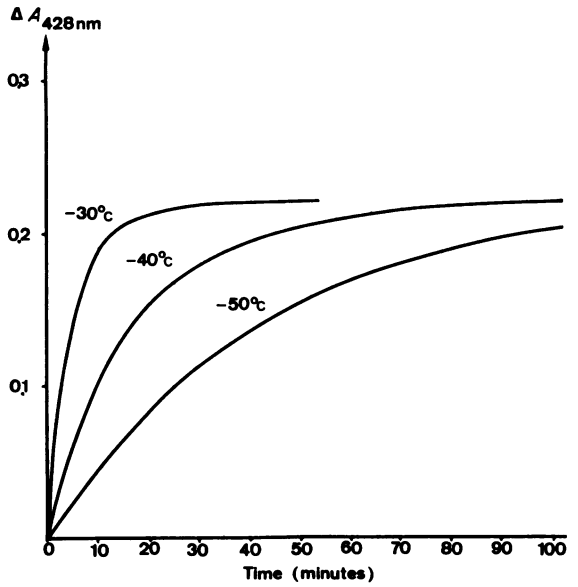


FIG. 2.—Kinetics of the conversion from *Complexes I* to *II* at various temperatures (same concentrations as for Fig. 1).

The Cotton effect appears again with Complex II, with a normal wavelength shift and a decrease in magnitude. This result is comparable to data obtained from various ligand forms of peroxidase.[13,14] Further information leading eventually to a structural interpretation of present data might be obtained by electron

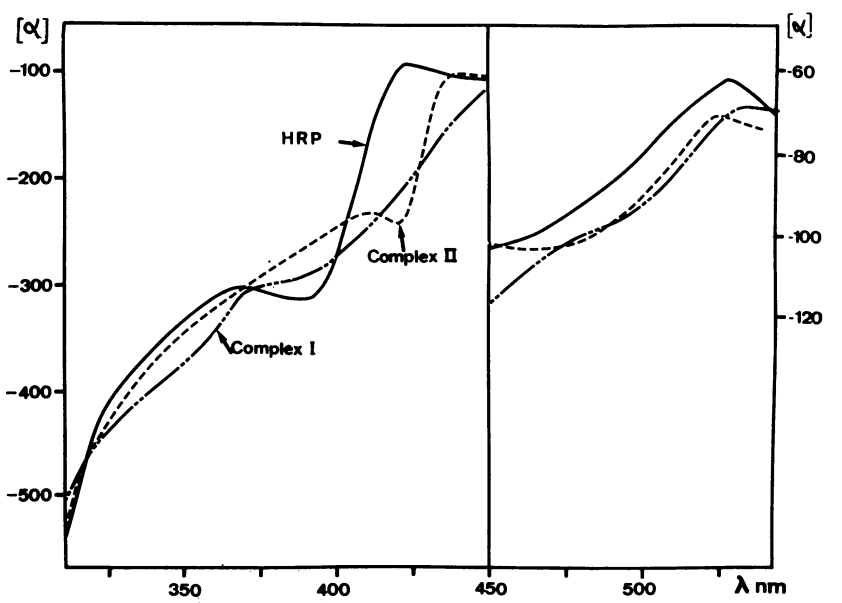


FIG. 3.—Optical rotatory dispersion spectra of the enzyme and of the *Complexes I* and *II* for Soret and visible bands. Temperature: −60°C; [α]: specific rotation. Final concentrations: *HRP*: $1.17 \times 10^{-5}$ M–$H_2O_2$: $1.75 \times 10^{-5}$ M.

spin resonance assay of the same samples. This is now under way and represents a new method of investigation of hemoproteins previously studied in frozen aqueous solutions.

**Conclusions.** From results obtained in the study of the intermediates encountered in catalyses by horseradish peroxidase, we can draw some general conclusions concerning the usefulness of the low temperature procedure applied to enzymic systems.

It should be possible to resolve the mechanism of a reaction into a number of discrete steps as a result of variation of energy of activation of the various steps as a function of temperature.

The quenching of reactions at a given intermediate stage permits not only the static (nonkinetic) analysis of the individual steps but also a study of kinetics between two consecutive steps with evaluation of the corresponding activation energy. It is therefore possible to start or stop reactions involving stoichiometric concentrations of enzyme and substrate without having to make the time scale of reactions experimentally accessible by lowering enzyme concentration and increasing the substrate concentration as in fast techniques. We can thus approach conditions found in nature more closely. The cooling-warming procedure can be used in such a way that a substrate can temporarily behave like an analogue undergoing only part of the catalytic reaction. In fact, the incomplete end product can be ultimately "activated" by a slight increase of temperature to give a completed end product.

Information about possible intermediates previously obtained only by using analogues and inhibitors might be obtained with normal substrates.

Many of the present fast techniques, which give nothing more than a characteristic relaxation time with no indication as to its significance, could be combined with the cooling-warming procedure, thus giving all the required structural information about intermediates—as in the present example of ORD spectra recorded for intermediates of the peroxidation reaction. Such studies may be complemented in the near future by electron spin resonance investigations now under way in this laboratory.[15] Other spectroscopic techniques, such as fluorescence, may be equally useful in the study of enzymic systems deserving more detailed static analysis, and there is no reason why x-ray diffraction should not be adapted to the cooling-warming procedure for the complete structural study of intermediates.

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