ABSORPTION SPECTRA OF PEROXIDASE SOLUTIONS

By

L. SZALAY and Z. VÁRKONYI

Institute of Biophysics, Attila József University, Szeged

(Received January 15, 1974)

In the absorption spectra of neutral tris-buffered solutions of $5 \cdot 10^{-8}$—$1 \cdot 10^{-5}$ M horseradish peroxidase, beside the known bands at 270—280 and 405 nm, a band at 190 nm is reported. The Beer—Lambert law of absorption is not valid, the molar extinction coefficients are concentration dependent even at the lowest concentrations investigated. The deviation from the Beer—Lambert law is explained by assuming the presence of aggregates. The extinction coefficient of the short wave band at 190 nm is $1.10 \cdot 10^6$ $l \cdot M^{-1} cm^{-1}$ in $2 \cdot 10^{-6}$ M solutions. Because of the invalidity of the Beer—Lambert law, the RZ-value (Reinheitszahl, extinction at 402 nm/extinction at 280 nm), generally used for characterizing the purity of peroxidase, is not unequivocal.

Introduction

Comparatively few data concerning absorption of peroxidase have been published until now. The earliest data are due to H. THEORELL and A. MAELY [1], who gave a relative absorption spectrum and proposed the ratio of the height of the band at 402 nm to the height of the band at 280 nm (the so-colled RZ-value) for characterizing the purity of peroxidase. K. G. PAUL [2] and L. M. SHANNON *at al.* [3] found significant changes in this ratio during purification of peroxidase. Also E. M. STRICKLAND [4] published absorption spectra, but without further explanation, as the emphasis in his investigations was on the study of circular dichroism spectra. The absorption spectra were measured in the wavelength range 250—450 nm, but the value of the molar extinction coefficient can be deduced only in some cases, for example at 401 nm, calculated from data of [3], it is $1.02 \cdot 10^5$ $l \cdot M^{-1}$ cm^{-1}.

As a criterion of purity was given on the basis of the absorption spectrum and this criterion, according to the investigations in [2] and [3] seems to be problematic, these spectra were redetermined and also the validity of the Beer—Lambert law for peroxidase solutions was studied. In addition, it seemed worth while to clear up the existence of an absorption band in the region below 250 nm, where an increase in absorption was found by earlier measurements.

Materials and methods

Lyophilized and 3× crystallized horseradish peroxidase of the Nutritional Biochemical Corporation (Cleveland) was used. The preparate was of 3170 units/mg activity and of RZ=3.0 purity. Tris-buffered solutions of pH=7.0 were prepared from the crystallized material by diluting a standard solution of $1 \cdot 10^{-5}$ M to the concentrations $5 \cdot 10^{-6}$, $2 \cdot 10^{-6}$, $1 \cdot 10^{-6}$, $5 \cdot 10^{-7}$, $2 \cdot 10^{-7}$, $1 \cdot 10^{-7}$ and $5 \cdot 10^{-8}$ M,

respectively. The solutions were stored at a temperature between 0 °C and 4 °C for about 24 hrs. before use.

Absorption spectra were measured partly with an Optica Milano (Type CF 4DR), partly with a Unicam (Type 1800) spectrophotometer. In a series of measurements made with $1 \cdot 10^{-5}$ M solutions the temperature dependence of the spectra in the temperature range 10—55 °C was also determined.

Results

The absorption spectra have three characteristic bands (Fig. 1—2). In the figures only curves of selected concentrations are presented. The maximum of the band in the ultraviolet is at 190 nm, that in the near ultraviolet between 270—280 nm and the third at about 405 nm. At 500 and 620 nm there are two shoulders (see [5]). The molar decadic extinction coefficients of the maxima in the region of shortest waves are about two orders of magnitude higher than in the two other regions ($110 \cdot 10^4$, $2.6 \cdot 10^4$ and $7.2 \cdot 10^4$ $l \cdot M^{-1}$ cm^{-1} in the order of increasing wavelengths of the locations of maxima in $2 \cdot 10^{-6}$ M solution). The absorption spectra are plotted with different scales according to the $\varepsilon(\lambda)$ values of the different spectral regions denoted by *A*, *B* (Fig. 1) and *A*, *B*, *C* (Fig. 2). The absorption spectra of the $2 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ M solution are, however, continued according to the scale of section *B* also in section *C*. The absorption is comparatively very low in the 250—450 nm

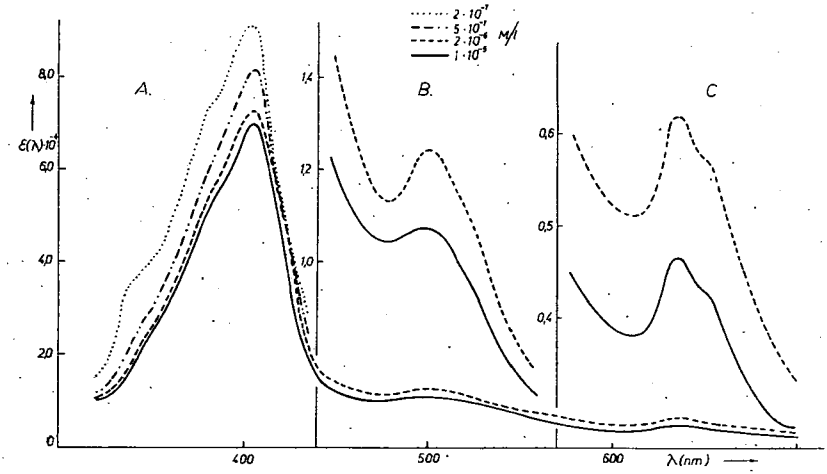

Fig. 1. Absorption spectra of neutral tris-buffered and water solutions of peroxidase as a function of concentration

spectral range. A similar technique was used in the 350—700 nm spectral region (Fig. 2).

Under 205 nm the absorption of the tris-buffered solution cannot be determined owing to the strong absorption of the buffer even in nitrogen atmosphere. Therefore, in this spectrum region the absorption of water solutions of peroxidase was measured and is shown in Fig. 1. *A*.

The molar decadic extinction coefficients are highly concentration dependent, revealing that the Beer-Lambert law of light absorption does not hold. This should be ascribed to physical or chemical interactions occuring between peroxidase molecules with increasing concentration.

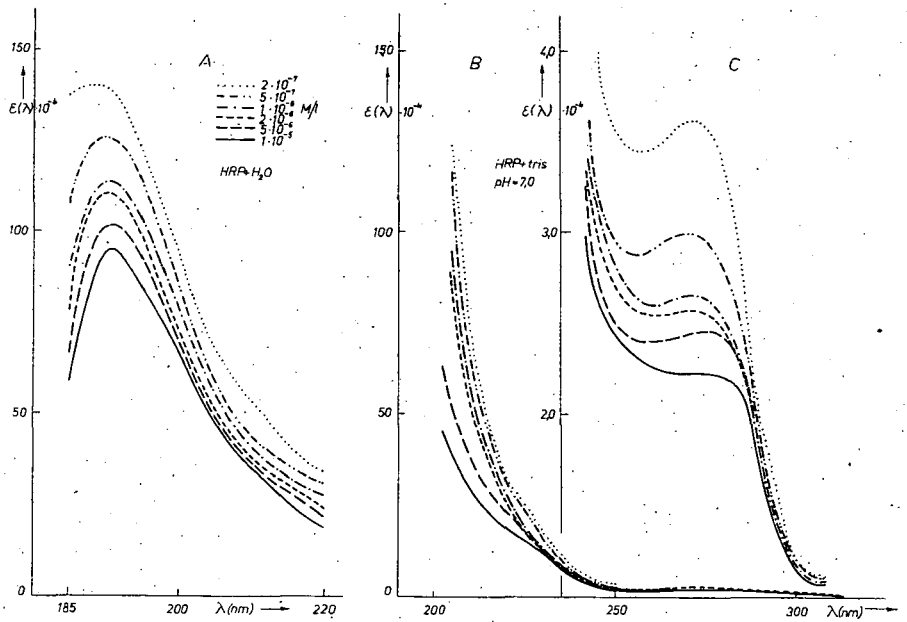

Fig. 2. Absorption spectra of neutral tris-buffered and water solutions of peroxidase as a function of concentration

Discussion

The absorption band at about 190 nm has not been published hitherto. This band of the spectrum corresponds partly to the absorption of the peptide bonds, partly to that of ring compounds [16]. The concentration dependence of the extinction coefficient was not described earlier. There are only few references to the Beer—Lambert law in treating the light absorption of proteins (see *e.g.* [1, 6]). According to our measurements the law is valid for the phosphate buffered (pH=7.0) solution of lysozyme in the $2 \cdot 10^{-4}$ M—$5 \cdot 10^{-7}$ M concentration range ($\varepsilon_{max}(\lambda)=3.5 \cdot 10^4$ $Ml^{-1}\,cm^{-1}$). Since the law is not valid for peroxidase, it seems questionable whether it is justified to define the purity of peroxidase on the basis of absorption spectra with the so-called RZ-value [1]. According to this definition, for pure peroxidase $RZ=\varepsilon(402)/\varepsilon(280)=3.04$. L. M. SHANNON and even H. THEORELL [1] found peroxidase preparations with RZ-values exceeding 3.04. Therefore, already L. M. SHANNON *et al.* [3] remarked that the use of the RZ-value is not satisfactory for characterizing the purity of the preparation. However, the critical remark was not

in connection with the Beer—Lambert law. In the case of our sample, RZ-values I. Since the absorption does not follow the Beer—Lambert law and the ratios of absorptions measured at different wavelengths changing with the concentration were found. These changes are presented in Table are concentration depe ndent, the RZ-value according to its original definition does not seem satisfactory for characterizing the purity of peroxidase.

Table I

Dependence of RZ-*values* $\left(\mathrm{RZ}=\frac{\varepsilon(402)}{\varepsilon(280)}\right)$ *on concentration*

C M/L	$\varepsilon(402)$ ($\mathrm{l \cdot M^{-1}\,cm^{-1}}$)	$\varepsilon(280)$ ($\mathrm{l \cdot M^{-1}\,cm^{-1}}$)	RZ
$2 \cdot 10^{-7}$	9.00	3.62	2.49
$5 \cdot 10^{-7}$	7.93	3.0	2.64
$1 \cdot 10^{-6}$	7.40	2.66	2.78
$2 \cdot 10^{-6}$	7.17	2.56	2.80
$5 \cdot 10^{-6}$	6.92	2.46	2.81
$1 \cdot 10^{-5}$	6.80	2.23	3.05

In some cases the deviation from the Beer—Lambert law can be explained by the appearence of associates. If, however, the molecules were spherical, an aggregation would be very difficult according to the following arguments. A spherical peroxidase molecule should have a radius $r=52$ Å, calculated from $r=1.67\ (M/d)^{1/3}$ with the molecular weight $\mathrm{M}=4 \cdot 10^4$ and density $d=1.3$ g $\mathrm{cm^{-3}}$. Supposing a random distribution of the spheres, from the formula $R^3=3(4\pi \cdot cN')^{-1}$ [8] (where c is the concentration in M/l, $N'=6 \cdot 10^{20}$ and R is the distance between two neighbouring molecules in Å), the average distance of neighbouring spheres in the $1 \cdot 10^{-5}$ M solution would be $R=326$ Å. If the distance between the centres of the spheres is 326 Å, the distance between the surfaces would exceed 200 Å; thus at this concentration no association could occur. Therefore, it should be supposed, that either the molecules can be better approached by ellipsoids, or some molecules are associated already during the dissolution of the crystalline material. Namely, with the axial ratio 12:1:1, neighbouring ellipsoids could come into contact in the solution mentioned above and the neighbouring molecules could form associates.

Approximate information concerning the form of the molecules can be obtained also by hydrodynamical methods. The constant β in the formula

$$M = \left[\frac{s \cdot N \cdot \eta_0}{\beta(1-\bar{v}d)}\right]^{3/2} \cdot [\eta_v]^{1/2}$$

is characteristic for the axial ratio [9]. M is the molecular weight of the protein, $\bar{v}$ the specific partial volume, η_0 the viscosity of the solvent, d the density of the solution, η_v the limiting viscosity, s the sedimentation constant, and $N=6\cdot10^{23}$. For tris-buffered solution of peroxidase, with $M=4\cdot10^4$, $\bar{v}=0.689\ \mathrm{cm^3\ g^{-1}}$, $s=3.5\cdot10^{-13}$ sec, $\eta_0=1$ cP, $\eta_v=1.087$ cP, $d=1.0012\ \mathrm{gcm^{-3}}$, $\beta=2.6\cdot10^6$ was obtained. The viscosities were determined at 25° C with a Höppler viscosimeter. The densities were measured, the other data were taken from [3] and [9]. According to H. A. SCHERAGA [10] the axial ratio of the ellipsoid for $\beta=2.6\cdot10^6$ is 15:1:1. With respect to the approximate character of the hydrodynamical methods, the accordance with our ratio (12:1:1) seems to corroborate the statement that the peroxidase molecule is rather well approximated by an ellipsoid.

As the Beer—Lambert law does not hold, the extinction coefficients obtained for different concentrations are only apparent. It can be supposed that in the $5\cdot10^{-8}$ M solution there are practically no aggregates. In this solution the extinction coefficients of the band maxima of the ring compounds are $\varepsilon(280)=3.6\cdot10^4$ and $\varepsilon(402)=8.5\cdot10^4\ \mathrm{l\cdot M^{-1}\ cm^{-1}}$, respectively. According to [12] $\varepsilon(280)=7\cdot10^4\ \mathrm{l\cdot M^{-1}\ cm^{-1}}$ and from [4] $\varepsilon(402)=10.8\cdot10^4\ \mathrm{l\cdot M^{-1}\ cm^{-1}}$. The deviations are due to differences in the preparations (in [4] A1 fraction was used).

The Beer—Lambert law is not valid even in 10^{-7} M solutions, in which the neighbouring molecules are in a distance of more than 1000 Å. Since the diluted solutions were prepared from a concentrated (in our case $1\cdot10^{-5}$ M) solution, if we assume that the aggregates formed in this solution are not completely disaggregated during dilution, this violation of the Beer—Lambert law can be understood. The validity of this explanation can be tested by studying the temperature dependence of the spectra. According to measurements in the temperature range 10—45° C the extinction coefficient at 280 nm increases only by a few percents. This means that the aggregates are not easily dissolved.

It seems unusual that the Beer—Lambert law is not valid for 10^{-7} M concentration; however, this case is not unique. It is known that in $1\cdot10^{-7}$ M dye solutions the Beer-Lambert law does not hold [13]. It was also concluded from fluorescence spectra that aggregates of pigment molecules may be present in similarly highly diluted solutions [15].

The deviation from the Beer—Lambert law may be in connection with the ratio of charged and neutral amino acids in peroxidase. Though the primary structure of peroxidase is not known in all details, the amino acid composition of the molecule is practically clarified [7, 14]; it consists of 60 charged and 250 neutral amino acids, the ratio of these numbers is 0.24. According to FISCHER [11] the minimum interaction energy originating from hydrophobic forces in water solutions is attained, if all charged amino acids are located on the surface of a sphere and the neutral ones in the interior. For a spherical particle a theoretical ratio $p=0.28$ is given. in [17] Since in our case the experimental p is smaller than the theoretical P_s, the surface is not completely covered by charged particles. Therefore, a decrease in the interaction energy of solute-solvent system association may occur and the molecular weight may depend on the concentration of the protein.

References

[1] *Theorell, H., A. Maehly:* Acta Chem. Scand. **4**, 422 (1950).
[2] *Paul, K. G.:* Acta Chem. Scand. **12**, 1312 (1958).
[3] *Shannon, L. M., E. Kay, F. Y. Lev:* J. Biol. Chem. **241**, 2166 (1966).
[4] *Strickland E. H.:* Biochim. Biophys. Acta **151**, 70 (1968).
[5] *Tamura, M.:* Biochim. Biophys. Acta **243**, 249 (1971).
[6] *Strickland, E. H., T. Horwitz, E. Kay, L. M. Shannon, M. Wilchek, C. Billups:* Biochemistry, **10**, 2631 (1971).
[7] *Welinder, K. G., L. B. Smillie, G. R. Schonbaum:* Can. J. Biochem. **50**, 44 (1972).
[8] *Förster, Th.:* Fluoreszenz organischer Verbindungen, Vandenhoeck und Ruprecht, Göttinge (1951). p. 177
[9] *Előd i P.:* Biológiai Tanulmányok (Biological Studies, in Hungarian) **2**, 33 Akadémiai Kiadó, Budapest (1966).
[10] *Scheraga, H. A., L. Mandelkern:* J. Amer. Chem. Soc. **75**, 179 (1953).
[11] *Fisher, B. F.:* Biochemistry **51**, 1285 (1964).
[12] *Flohe, L., E. Schaick, W. Voelter, A. Wendel* Hoppe-Seyler's Z. Physiol. Chem. **352**, 170 (1971).
[13] *Rabinowitch, E., L. F. Epstein:* J. Amer. Chem. Soc. **63**, 69 (1941).
[14] *Welinder, K. G., L. B. Smille:* Can. J. Biochem. **50**, 63 (1972).
[15] *Singhal, S., L. Szalay, E. Tombácz:* Acta Phys. et Chem. Szeged **19**, 11 (1973).
[16] *Preiss, J. W., R. Setlow:* J. Chem. Phys. **25**, 138 (1956).

АБСОРБЦИОННЫЙ СПЕКТР РАСТВОРА ПЕРОКСИДАЗЫ

Л. Салаи, З. Варкони

В нейтральных „трис"-буферных растворах пероксидазы, полученной из хрена пр концентрациях $5 \cdot 10^{-8} - 1 \cdot 10^{-5}$ моль/л в абсорбционных спектрах обнаружена, кроме известных 270—280 и 405 нм полос поглощения, новая полоса при 190 нм. Закон Ламберта—Бера не удовлетлетворяется ни в одном случае для этих полос, молярные экстинкционные коеффициенты показывают концентрационную зависимость даже при самых больших разбавлениях. Отклонение от закона Ламберта—Бера может объясняться образованием ассоциатов. Коеффициент экстинкции при поглощении на 190 нм составляет для раствора концентрации $2 \cdot 10^{-6}$ моля $1{,}1 \cdot 10^{6}$ л. м.$^{-1}$. см.$^{-1}$ Вследствие неприемлености закона Ламберта—Бера, обычно применяемое RZ-число (=экстинкция при 402 нм/экстинкция при 280 нм) не является однозначной характеристикой.