Fluorescence Studies on Ferredoxin-NADP Reductase

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With highly purified preparations of ferredoxin-NADP reductase (EC 1.6.99.4; also EC 1.6.1.1) from spinach chloroplasts a strong flavin fluorescence was observed (excitation at 450 nm). At pH 7.0, the fluorescence was approximately 40% higher than that of free FAD. Pure reductase from the heterokont alga *Bumilleriopsis* had only a fluorescence of less than 5% of that of the spinach protein. Essentially the same low fluorescence was found with impure spinach reductase preparations. The low-fluorescent samples of both species exhibited about the same quenching response to salts and buffers which differed from the highly fluorescent spinach flavoprotein preparation.

The low fluorescence of the algal protein could be enhanced by NADP, but not by ferredoxin. Maximum fluorescence increase was observed when reductase and NADP were present in a 1:1 molar ratio. Ferredoxin did not counteract the effect. Previous studies on binding sites of the reductase are hereby substantiated.

Introduction

Ferredoxin-NADP reductase is the flavoprotein operating as the terminal protein redox carrier in the photosynthetic electron transport chain. It has at least two binding sites and is, therefore, able to react with ferredoxin on the one hand and with NADP on the other. Initially, its physiological role was obscured by the presence of additional enzymic activities, namely, diaphorase and transhydrogenase [1, 2]. The role of these latter reactions was unclear for several years after their discovery, but recent data indicated that they are associated with physiological photosynthetic NADP reduction [3, 4]. The reductase has been characterized by a thorough study of its complex formation with ferredoxin which is accompanied by certain changes in enzymic activity [5].

It was the purpose of the present investigation to characterize further the properties of the reductase by means of fluorescence studies. Of particular interest was the fluorescence response of the spinach flavoprotein (of *Spinacia oleracea*) to its natural substrates ferredoxin and NADP. Furthermore, we wished to compare the protein from spinach chloroplasts with that from *Bumilleriopsis filiformis* Vischer, a heterokont alga [6, 7].

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Materials and Methods

Ferredoxin-NADP reductase (EC 1.6.99.4; also EC 1.6.1.1) from spinach was prepared according to a slightly modified procedure of Forti [8]. The hydroxyapatite (Bio-Gel HTP) column was 1.5×10 cm (step 4A in ref. 8) and equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. About 11,000 units of activity (diaphorase assay using potassium ferricyanide, see also ref. 8 for definition of units) were applied to the column and 8000 units were recovered. However, only about one-half of the protein recovered exhibited a ratio of E_{274}/E_{458} of about 9 (*cf.* ref. 5). A sample with a ratio of 18 was used for comparative purposes. The reductase from *Bumilleriopsis filiformis* was prepared as previously described [5, 6], but an additional (second) step on Sephadex G-50 (3×30 cm column) preceded the Sephadex chromatography on G-75 (2×10 cm column).

The protein was concentrated with Aquacide II (Calbiochem, Los Angeles, Calif., USA) when necessary. The extinction ratio E_{274}/E_{458} was 7.8; the protein, therefore, was considered to be of highest purity (see Fig. 1 of ref. 5 for spectrum). The reductases were stored a couple of months in the deep-freeze (-25 °C) in 0.01 M Tris, pH 7 to 8, before use, which caused some loss of activity (not

Abbreviations: E. extinction (optical density); the subscript indicates wavelength F; d, ferredoxin (from *Bumilleriopsis* or spinach); Fp-Sp, Fp-B, ferredoxin-NADP reductase from spinach and *Bumilleriopsis*, respectively; Tris, tris-(hydroxymethyl)-aminomethane (buffer, adjusted with HCl); Mes, 2-(*N*-morpholino-)ethanesulfonic acid (buffer, adjusted with NaOH).

Table I. Influence of pH on flavin fluorescence peak height of FAD and ferredoxin-NADP reductase. Data represent peak heights of transmitted fluorescence at 524 nm and are expressed as units on the recorder (chart) referred to a FAD or flavoprotein concentration corresponding to an optical density of 1.0 at 458 nm. Total chart width equals 100 recorder chart units. Amplifier stetting is 0.01 with sensitivity at 50. Hitachi recorder span is 50 mV. See "Materials and Methods" for details.

pH*	FAD	Fp-Sp Prep. I $E_{274}/E_{458} = 9.3$	Fp-Sp Prep. II $E_{274}/E_{458} = 18$	Fp-B Prep. I $E_{274}/E_{458} = 7.8$	Fp-B Prep. II $E_{274}/E_{458} = 8.0$
8.0	475	590	—	23.0	—
7.0	485	670	38.6	23.2	128
6.0	612	730	—	26.4	—
5.0	693	790	—	34.0	—
4.0	1220	1032	—	37.7**	—

* From pH 8 to pH 6: Mes-NaOH buffer; pH 5 and 4: Sodium citrate/potassium phosphate buffer; all 0.1 M.
** Slight turbidity after some minutes of incubation.

more than 30%), equal for *both* the spinach and the algal reductase preparations. Diaphorase activity was determined with dichlorophenol indophenol (0.2 mM) and 0.2 mM reduced NADP in 0.075 M Tris, pH 8; transhydrogenase activity was measured at pH 8 in 0.075 M Tris, as described[5]; NADPH was approx. 0.02 mM and was constantly regenerated by glucose-6-phosphate (1 mM) and excess glucose-6-phosphate dehydrogenase; see Forti and Sturani[9]. Activities were expressed as the change of optical density at 610 and 340 nm, respectively, during a 10 sec experimental time. The ratio of diaphorase to transhydrogenase activity, expressed in these units, was 99 for the spinach reductase preparation I (Fp-Sp, prep. I; $E_{274}/E_{458} = 9.3$). It was 200 for both reductase preparations from *Bumilleriopsis* (Fp-B; $E_{274}/E_{458} = 7.8$ and 8.0, see Table I). These activity ratios can be computed also from earlier reports of reductases checked immediately after their preparation (see Table I of ref. 5).

Spinach reductase preparation II (Fp-Sp, prep. II; $E_{274}/E_{458} = 18$), which was prepared deliberately less pure than preparation I, had a ratio of diaphorase to transhydrogenase activity of 183. This was due, as would be expected, almost exclusively to an increased diaphorase activity, which was twice as high as that of preparation I, whereas transhydrogenase activity was the same. All enzymatic activities were based on the same flavin absorbance (see ref. 5).

Fluorescence measurements were made with an Aminco-Bowman Spectrofluorimeter (American Instruments Inc., Silver Spring, Md., USA; Cat. No. 4-8106). An XY-recorder of Electro-Instruments, San Diego, Calif. (Model 101-1519) was used to plot the fluorescence spectra (from 470 to 650 nm). A Hitachi recorder (Model 165; 0 to 10 V range) was used to measure the intensity of fluorescence at a fixed wavelength (524 nm). A 50 mV span setting gave an additional 2 times amplification of the ordinate data compared with those of the XY-recorder mentioned above. The readings could be zeroed (at start) by a zero suppression attachment (from Coleman-Hitachi). The actinic (exciting) light monochromator was set at 450 nm with a 10 nm half-bandwidth, the fluorescence monochromator had a 20 nm half-bandwidth. Settings of the amplifier are noted in the legends. Scanning speed of the fluorescence monochromator was as slow as possible. Protein concentrations were 4 to 16 μM for the *Bumilleriopsis* protein, and 0.6 to 1.2 μM for the spinach reductase. Reaction volume was 1 ml.

Before either the first reading or the fluorescence spectrum was taken, the mixture was allowed to equilibrate for 3 min when the spinach protein was used, and 1 min for the algal reductase. Reaction temperature was kept at 23 to 24 °C (room temperature) by placing a strong feather fan underneath the cuvette chamber. Slight changes in fluorescence intensities were observed within the reaction time (*cf.* Fig. 3).

Care was taken to use fresh glass-distilled water only. The instrument was calibrated for correct wavelength readings as indicated by the manufacturer (see also Chen[10]). No correction of the fluorescence spectra according to phototube sensitivity was made.

NADP (Na-salt, SIGMA grade) was from Sigma, St. Louis, Mo.; NAD from P-L Biochemical, Milwaukee, Wisc.; buffers and salts from Baker Chemical Co., Philipsburg, N. J. and were analyzed reagents.

Results

Fluorescence spectra of the reductases are given in Fig. 1 and compared with FAD in 0.1 M Mes-buffer, pH 7.0. The peak of all three spectra is at approx. 524 nm. All three spectra shown are based

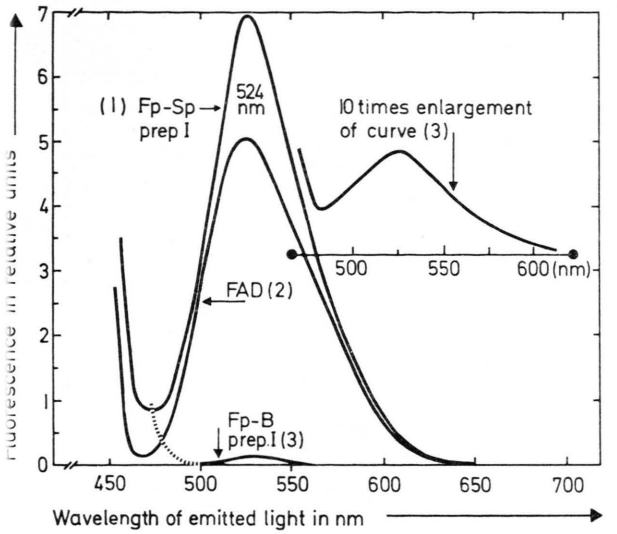


Fig. 1. Flavin fluorescence spectra of FAD and ferredoxin-NADP reductases. Excitation at 450 nm in 0.1 M Mes-buffer, pH 7.0; all spectra calculated for 5.7 μM FAD content. One (relative) unit is 25 mm on the Y-scale of the XY-Aminco recorder with the (photomultiplier) microphotometer range set at 0.003 × 50 (sensitivity).

on the same flavin content (for the molar extinction coefficient, see Whitby [11]).

The interesting result is that, firstly, the FAD fluorescence of each reductase is very different. The very high emission of the spinach protein is contrasted with the very low fluorescence of the algal protein. Secondly, the fluorescence of the spinach protein is higher than that of free FAD under the same conditions; with Mes-buffer (pH 7.0; 0.1 M), the purified spinach reductase has a 37% stronger fluorescence than FAD. It is 46% higher when the concentration of the Mes-buffer is lowered to 0.05 M.

Quantitative data additional to these findings is given in Table I. Both reductases and FAD exhibited increasing fluorescence by lowering the pH to 4 (and below). The pH, however, cannot be decreased further because of turbidity which gives an increased light scattering and masks the low fluorescence of the *Bumilleriopsis* reductase. However, it is evident that the increase of fluorescence as the pH is lowered from 8 to 5 is comparable for free FAD and the reductases (between 34 and 48%). Within this pH range the enzyme is stable during the experimental time; the fluorescence increase was reversible. According to Bessey *et al.* [12], this increase cannot be observed with FMN (see also ref. 13). It is suggested that the reductases contain FAD and not FMN as was well documented before for the spinach protein [1, 14] and that from *Chlorella* [15]. The same is now true for the protein from *Bumilleriopsis*.

Furthermore, as seen in Table I, the high fluorescence of the spinach protein is related to, though not directly, the state of purity. Spinach reductase preparation II, which is approx. 50% pure (based on the E_{274}/E_{458} ratio) has only a fluorescence of 39 as compared to 670 for the more pure preparation (expressed in relative fluorescence units). It is seen further that the fluorescence of the algal protein is somewhat variable; 23 abd 128 in rel. units with two preparations of a comparable high degree of purity as indicated by the low ratio of E_{274}/E_{458} (Table I).

Both reductases and FAD yield the same intensity of flavin fluorescence when the protein component was denatured with urea in acid medium (Table II). *Bumilleriopsis* reductase is apparently more stable than the spinach protein, since with both *Bumilleriopsis* preparations urea denaturation does not increase the fluorescence completely to the intensity of free flavin, as is the case with spinach reductase.

Mes-buffer, pH 7, was used in most experiments described herein since it has no denaturing effect on the reductase. This buffer causes some increase of fluorescence during longer experimental times (see

Exp. No.	Additions	Mes-buffer 0.05 M, pH 7.0 FAD	Citric Acid, 0.1 M* FAD	Fp-Sp Prep. I	Fp-B Prep. I/II
1	—	558	2440	2400	2100/1920**
2	0.28 M Urea	1130	—		
3	0.40 M Urea	—	3840	3720	3160/3160
4	0.53 M Urea	2100	4180	4140	3480/3540
5	0.60 M Urea	—	4540	4340	3600/3720
6	0.53 M Urea without FAD	11.8			—

Table II. Flavin fluorescence under the influence of urea. Data are expressed as described in the legend of Table I.

* pH is 2.1 in Exp. No. 1 and increases to 3.2 in Exp. No. 5 corresponding to inreasing urea concentration.

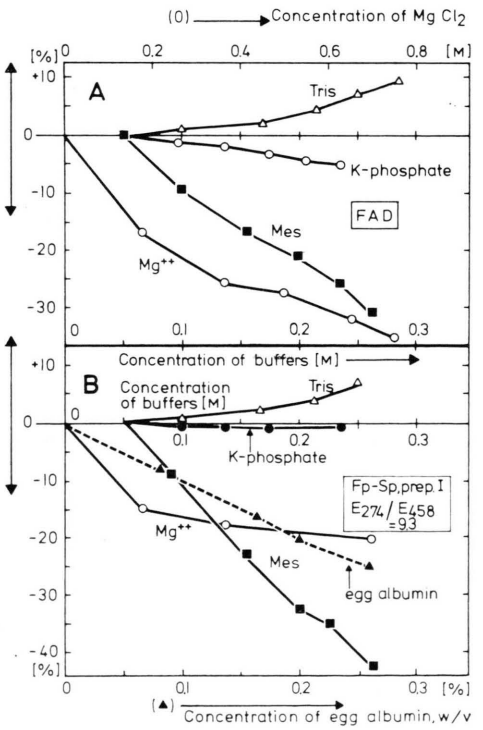
** Figures for *Bumilleriopsis* reductase preparations I and II, respectively.

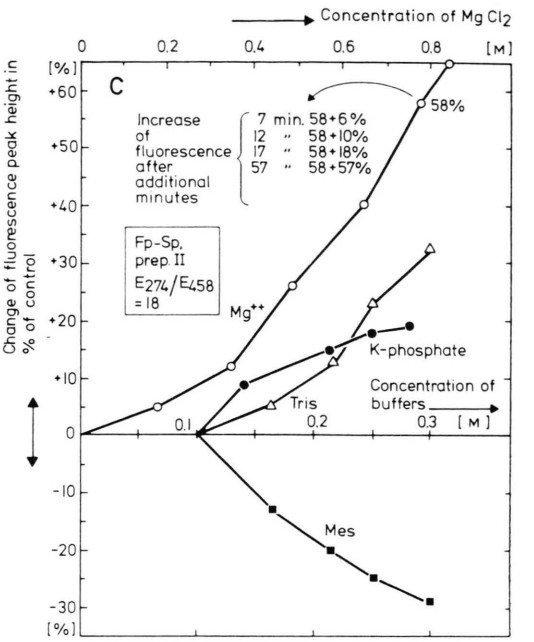
Fig. 3). It is negligible with 0.1 M concentration, but increases considerably when the concentration is doubled and tripled.

The highly *purified* preparations from spinach and *Bumilleriopsis* differ very much in flavin fluorescence intensity. The *impure* preparation from spinach, on the other hand, resembles the pure algal protein since with both of them fluorescence is very low (see Table I).

The experiment presented in Fig. 2 corroborates this by measuring the fluorescence of FAD and both flavoproteins under the influence of increasing buffer and Mg^{++} ($MgCl_2$) concentrations. In this respect, the pure reductase from spinach resembles very much free FAD. In both cases, a higher concentration of Mes-buffer and magnesium ions decreases fluorescence. Phosphate has little effect, so has Tris, although with Tris a small fluorescence increase is observed. When similar concentration experiments were performed with the *impure* spinach reductase, results different from those of the pure spinach preparations, but qualitatively very similar to those with the purified *Bumilleriopsis* protein, were obtained (Figs 2 C and D). Mes-buffer decreases fluorescence as measured before. Magnesium ions, on the other hand, increase it, as does potassium phosphate.

Egg albumin (Sigma, cryst.) has comparatively little effect on the fluorescence decrease with the pure spinach reductase (Fig. 2 B). Albumin was added in





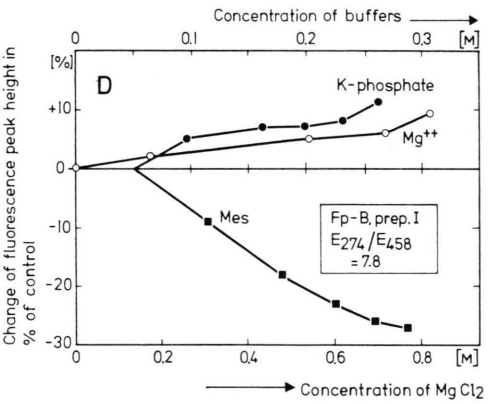


Fig. 2. Change of flavin fluorescence of FAD and Fd-NADP reductases by increasing buffer and $MgCl_2$ concentrations. The buffer concentration was kept constant 0.1 M, pH 7.0 when the $MgCl_2$ concentration was varied (○—○). Indications on the abscissa refer to both Figs. 2 A and 2 B. In Fig. 2 C the time course of fluorescence increase in 0.76 M $MgCl_2$ in indicated (see arrow). Instrument settings: 0.001 × 50 (see Fig. 1).

twenty-fold excess of reductase protein. The increase of fluorescence by magnesium ions is time dependent; the data plotted in Fig. 2 C were taken 3 min after adding the Mg^{++} concentrations indicated.

These very striking differences in the fluorescence properties of both highly purified reductases from spinach and *Bumilleriopsis*, respectively, are further documented in the following experiments, which, in addition, extend our previously published studies of the binding sites of the reductase [3, 4].

Adding NADP to the pure spinach reductase causes no increase of its already strong fluorescence; with the reductase from the alga, the fluorescence increases. (Since the basal fluorescence of the free algal protein is low, the meter has to be carefully adjusted and operated at the highest amplification. For protein concentration, see "Materials and Methods".) A titration of the algal reductase with NADP up to and beyond the point where maximum fluorescence increase is attained is shown in Fig. 3. By subtracting the increase increments from the time course of the control (determined in a separate experiment), these differences can be plotted against NADP concentration. As seen in the figure, the saturation level is reached when both reactants are present in a 1:1 molar ratio (see inset of Fig. 3).

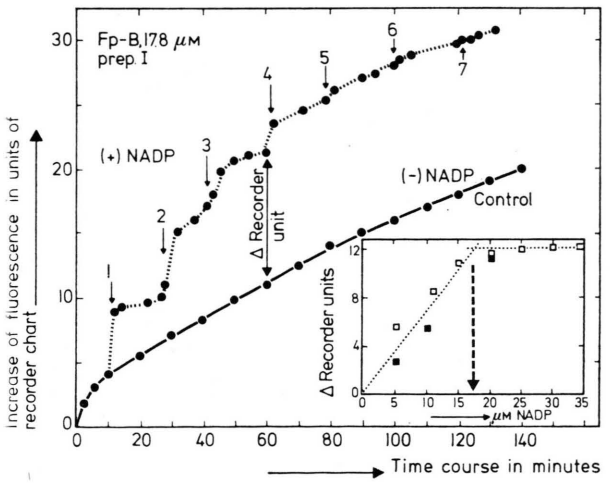


Fig. 3. Increase of flavin fluorescence during titration of the algal Fd-NADP reductase with NADP. Five μl of 1 mM NADP was added (per 1 ml of original reaction mixture in 0.1 M Mes, pH 7.0) at the time intervals indicated by numbers 1, 2, 3, ... The difference of fluorescence units between the sample with NADP added and the control without NADP are plotted in the inset *vs* the concentration of NADP present. Fluorescence measured at 524 nm. Instrument settings: 0.001 × 50 (see Fig. 1). Hitachi recorder span 20 mV with readings suppressed to zero at start. Chart speed 5 mm/min.

Ferredoxin, on the other hand, has no effect on the fluorescence of the flavoproteins, even though the proteins were from the same species. This was not expected. However, with NADP present in the reaction mixture, at a concentration saturating for the fluorescence increase of the *Bumilleriopsis* reductase, ferredoxin does not counteract the observed effect of fluorescence increase. In fact, it even enhances it somewhat, provided the ferredoxin concentration is not too high (Fig. 4).

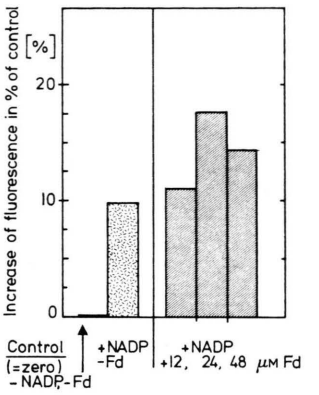


Fig. 4. Increase of flavin fluorescence by NADP of the Fd-NADP reductase from *Bumilleriopsis* in the presence of ferredoxin. In 0.05 M Mes, pH 7.0; NADP 50 μM; reductase 8.9 μM (prep. I); for instrument settings, see legend of Fig. 3.

Discussion

Generally, flavoproteins do not show the intense fluorescence of the free FAD (or FMN). In fact, fluorescence is often very low or almost non-detectable (for review see Palmer and Massey [16]). Lipoyl dehydrogenase is a well-known example to the contrary [17]. Here, the flavoprotein (at 5 °C) has a fluorescence peak at 520 nm which is about 5 times higher than that of the free FAD. It may be assumed that the protein exerts a strong conformational change in the FAD, thereby preventing fluorescence by closing the FAD molecule to the "hairpin" configuration which is low-fluorescent [13, 18]. This internal stacking complex of the free flavin can also be opened to a more fluorescent form by dissolving the FAD in certain nonpolar solvents [19]. It is evident that FMN does not show this effect. In the flavoproteins with very little fluorescence the flavin may also be quenched by tryptophan or tyrosine residues of the protein [13, 16].

Spinach reductase was reported to be non-fluorescent [20]. Our data show, indeed, the correctness of this observation provided the protien used is not highly purified. In the case of *Bumilleriopsis* reductase, even the highly purified form does not show a substantial fluorescence. Since egg albumin in a 20-fold excess does not decrease fluorescence dramatically (see Fig. 2 B), it might be concluded that a more specific protein component is responsible for the strong fluorescence quenching observed with the preparation which is only 50% pure Table I; Fp-Sp, prep. II). It is tentatively assumed, therefore, that in case of the purified highly fluorescent spinach reductase a quenching factor was removed, whereas with the algal reductase such a quenching factor may remain even though the enzyme has been brought to a very high degree of purity. If such an assumed factor does indeed exist, it should be one of low molecular weight, since it does not contribute substantially to the height of the protein absorbance peak (see Table I).

The highly fluorescent spinach redcutase preparation could not be enhanced by NADP. In contrast, the low-fluorescent algal reductase was. Here, the strongly quenched FAD group could be influenced by the nucleotide; the total change, however, was only approx. a 15% rise of fluorescence which could be accurately determined only under the conditions mentioned.

The finding is in good agreement with former enzymological observations [3, 4]. Firstly, the maximum increase in fluorescence occurs at a 1:1 molar ratio to NADP to protein. So, there is only one binding site for the nucleotide on the protein provided the nucleotide is added in low concentrations, as indicated in Fig. 3. From previous studies, at least two binding sites on the reductase were proposed: One for the nucleotide(s) which are NAD, NADP or thionicotinamide-NADP, and one for ferredoxin. (Inhibitor studies imply that in addition a third site is present. Binding at this third side affects the interaction of the enzyme with ferredoxin [21].) Only when the nucleotides are present in high concentrations can the ferredoxin site also be occupied by nucleotides (this was demonstrated with NAD as *H*-acceptor in the NADP : NAD transhydrogenase assay. NAD was present in approx. 650-times the concentration of the reductase [5]. In this case, NAD could be bound at the ferredoxin site of the enzyme).

Secondly, the data show that ferredoxin does not interfere with NADP binding to the flavoprotein, since the fluorescence increase by NADP is not counteracted by higher ferredoxin concentrations. This was expected from previous studies which showed that ferredoxin quite specifically occupies just one site on the enzyme, even when present in very high concentrations as compared to that of the reductase [5]. Although ferredoxin itself does not change the fluorescence of the free protein, it has a slight enhancing effect when NADP is present. This supports the conclusion that both ferredoxin and NADP are bound to the enzyme at separate sites [3].

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