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Perspectives in Biochemistry

Trends in Biochemistry and Enzymology of Cellulose Degradation

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Cellulases are the group of hydrolytic enzymes able to hydrolyze insoluble cellulose to glucose. They are produced by microorganisms, plants, and animals (in this case by symbiotic microorganisms), usually as a cellulase system of several distinct enzymes. There are three types of enzymes that have been traditionally assigned to the cellulase system: endoglucanases (endo-1,4-β-glucanases or 1,4-β-D-glucan 4-glucanohydrolases, EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-glucanases or 1,4-β-D-glucan cellobiohydrolases, EC 3.2.1.91), and cellobiases (β-glucosidases or β-D-glucoside glucohydrolases, EC 3.2.1.21). All of the principal enzymes of the cellulase system have been purified to homogeneity in numerous laboratories over the last 15–20 years.

Cellulolytic enzymes isolated from various sources differ in their molecular characteristics (molecular weight, amino acid composition and sequence, isoelectric point, carbohydrate content), adsorbability on to cellulose, catalytic activity, and substrate specificity. Furthermore, cellulase components from almost any organism occur as a number of multiple forms. All this leads to a literally infinite variety of differing cellulase systems from various sources, differing in their composition and catalytic characteristics of the enzymes in the hydrolysis of cellulose and its numerous morphological and chemical derivatives.

Some cellulases, particularly of bacterial origin, are known to be strongly associated with microbial cells (and apparently perform their catalytical function in vivo in an "immobilized" state) in contrast to the abundantly described extracellular fungal cellulases. Some cellulases are organized in supramolecular structures, cellulosomes, found for some bacterial cellulases, in contrast with many other multicomponent cellulase systems apparently lacking spatial organization. Cellulase systems often show only two or three individual components (including at least one endoglucanase), but in a number of cases thorough resolution of a cellulase system from a single source revealed 15–20 or more individual components (Hayn & Esterbauer, 1985; Knowles et al., 1987; Sprey, 1988). Fungal cellobiohydrolases sometimes show a marked tendency to form aggregates with endoglucanases, and those aggregates are extremely difficult to break up into component parts (Wood et al., 1989).

Synergism between the individual components of a cellulase system acting toward insoluble cellulose adds further complexities to the study of the mechanisms of action of cellulases; i.e., the action of a mixture of two or more individual cellulolytic components is greater than the sum of the action of each component. The main problem in studying this phenomenon is that the synergistic effect varies depending on which of the multiple forms of the cellulolytic components have been used in the study, on the source of cellulases, and on the cellulose sample (amorphous or crystalline, to mention the two extreme variants) used for the experiment. Definitive quantitative parameters regarding these points have not been found up to the present time.

It is now more apparent that the mode of action of fungal cellulases and that of bacterial cellulases differ significantly, at least in vivo. Hydrolysis of cellulose by bacterial cellulases apparently occurs at sites where contact between bacterial cells and the cellulose surface is made. This takes place under the action of structurally ordered multicomponent formations (cellulosomes), bound to the cell wall of the bacteria and organized in turn into catalytically active "protuberances" (Lamed & Bayer, 1986). If so, it may be that extracellular bacterial cellulases (detected in a cultural media in vastly lower quantities compared with fungal cellulases) are only "splinters" of cellulosomes and polycellulosomes. Thus, their study might only give indirect notions regarding the mechanism of the degradation of cellulose by bacterial cellulases.

It might occur at first sight that a study of cellulases (a system that requires so much characterization of both the multicomponent cellulase complex and the cellulosic substrate) may be impossible and that the destiny of this area is an

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empirical collection of experimental facts. Indeed many published studies appear as a mass of data, difficult to interpret on a mechanistic level. Even so, a number of fundamental questions of the biochemistry and enzymology of the hydrolysis of cellulose beg resolution. These questions include the following.

(1) Why are some cellulase systems capable of hydrolyzing both *crystalline* and *amorphous* cellulose while others can only degrade *amorphous* cellulose?

(2) Why do some cellulolytic enzymes (endoglucanases and cellobiohydrolases) show synergism with some isoenzymes and yet do not show it with others, although the "classified" substrate specificity may be the same for both groups of the isoenzymes? Why in several cases do cellobiohydrolases show synergism with endoglucanases from some sources and yet do not show it with same type of enzyme from other sources? What is the reason for the so-called "exo–exo" synergism (that is, the mutual enhanced catalytic action of two cellobiohydrolases)?

(3) What are similar and different in the mechanisms of cellulose hydrolysis by cellulases of *bacterial* and *fungal* origin?

(4) Why do cellulolytic enzymes differ in their affinity for cellulose? [The range of the values of the adsorption constants for different endoglucanases reaches a thousand times (Rabinovich et al., 1983; Klyosov et al., 1987)]. How does this difference in the adsorption capacity reflect on the catalytic activity of the enzymes?

(5) How do adsorbed cellulases behave on the cellulose surface? Does a *multiple* attack on cellulose by the adsorbed cellulases (endoglucanases and/or cellobiohydrolases) take place without their desorption from the substrate's surface, or does a cellulolytic enzyme desorb after each scission in order to bind then with another site of cellulose?

(6) What is the reason for the phenomenon of defibrillation of cellulose (a short fiber formation) under the action of certain cellulases? What causes it and what does it lead to? Why do some cellulases show this *mechanochemical* effect on cellulose whereas others do not?

(7) How can glucose be formed from cellulose under the action of an individual *endoglucanase*?

These questions have not been satisfactorily answered to date but one way or another have been the subject of discussion in many publications. The solution of any of these questions would be a major step forward in understanding the mechanisms not only of the enzymatic conversion of cellulose but also of the biological degradation of biopolymers in general.

Summarized below are some new ideas and experimental data that focus toward a solution to the questions posed above.

WHAT DIFFERENTIATES "FULL-VALUE" CELLULASE SYSTEMS FROM "LOW-VALUE" ONES?

It was well-known that cellulase systems from some biological sources are able to hydrolyze both amorphous and *crystalline* cellulose ("complete", "full-value", or "true" cellulases), whereas other cellulase systems are active only toward amorphous cellulose ("low value"). The latter are practically inactive toward crystalline cellulose (the degree of conversion does not usually exceed 2–5% and can easily be explained as the hydrolysis of the amorphous fraction of cellulose).

Figure 1 shows how crystalline cellulose distinguishes between low-value and full-value cellulase systems. It is seen that the transition from amorphous to crystalline cellulose, with a degree of crystallinity of 60–70 or higher, leads to a rapid increase of the ratio of the hydrolysis rates for a complete system (e.g., from *Trichoderma reesei*) to a low-value system (e.g., from *Sporotrichum dimorphosporum*) (Klyosov et al., 1982).

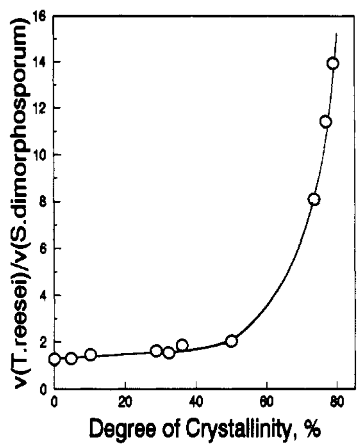

FIGURE 1: Relative reactivity of a full-value and a low-value cellulase complex (from *T. reesei* and *S. dimorphosporum*, respectively, in terms of the ratio of their stationary velocities) toward cellulose of different degrees of crystallinity. The open point on the vertical axis corresponds to soluble (carboxymethyl)cellulose (Klyosov et al., 1982).

Three principal hypotheses, any of which can explain the efficiency of full-value cellulase systems toward crystalline cellulose, are formulated as follows: (i) full-value cellulase systems contain a *cellobiohydrolase* (Eriksson & Pettersson, 1975; Gow & Wood, 1988); (ii) they contain an *endoglucanase* which adsorbs *tightly* on cellulose (Rabinovich et al., 1981, 1983; Klyosov et al., 1982, 1983, 1986, 1988; Chernoglazov et al., 1983); (iii) they contain an *endoglucanase* able to produce *glucose* as one of the major products resulting from the hydrolysis (Klyosov et al., 1987).

All these hypothesis are based on experimental data. At the same time, there is no indication that these hypothesis are alternative ones. In other words, it might be that a full-value cellulase system of a minimal composition can contain two enzymes only—cellobiohydrolase with certain properties (it is left to be revealed which properties) and endoglucanase tightly adsorbed on cellulose and producing noticeable amounts of glucose during the hydrolysis of cellulose. It is possible also that the biosynthesis of such full-value cellobiohydrolase and endoglucanase is somehow coordinated in certain microorganisms, and these very microorganisms produce complete cellulase systems.

These three hypotheses are supported by the following experimental data.

(i) All the complete or full-value cellulase systems which have been studied so far (e.g., those from *Trichoderma reesei*, *Trichoderma viride*, *Trichoderma koningii*, *Sporotrichum pulverulentum*, *Penicillium pinophilum*, *Fusarium solani*, *Fusarium lini*, and *Sclerotium rolfsii*) indeed contain cellobiohydrolases (Eriksson & Pettersson, 1975; Wood & McCrae, 1986a,b; Lachke & Deshpande, 1988; Gow & Wood, 1988; Wood et al., 1988). The cellobiohydrolases reinforce the catalytic action of endoglucanases from the same systems by acting synergistically with them. This in turn leads to an increase in the overall cellulolytic activity of the system, apparently making it a full-value one. But the presence of a cellobiohydrolase in cellulase systems might not be the only factor necessary for making the system complete. Thus, the addition of cellobiohydrolases from *T. koningii* or *F. solani* to low-value cellulase preparations from *Myrothecium verrucaria*, *Stachybotrys atra*, *Gliocladium roseum*, *Memnoniella echinata*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, or *Bacteroides succinogenes* does not lead to synergism and

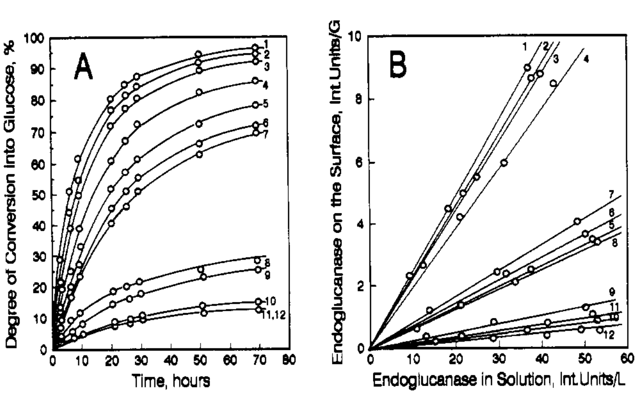

FIGURE 2: Hydrolysis of microcrystalline cellulose by cellulase complexes (panel A) and the adsorption capability of the complexes on microcrystalline cellulose (panel B). Source of the complex: (1) *T. reesei*, (2) *T. viride*, (3) *Trichoderma longibrachiatum*, (4) *Geotrichum candidum*, (5) *Trichoderma lignorum*, (6) *Aspergillus terreus*, (7) *T. koningii*, (8) *M. verrucaria*, (9) *S. dimorphosporum*, (10) *T. viride* (Boehringer Mannheim), (11) *A. niger*, and (12) *A. foetidus*. The hydrolysis was performed with an excess of cellobiase. The slopes in panel B correspond to the partition coefficients (K_p) of endoglucanases between the substrate surface and the water phase (Rabinovich et al., 1981).

to the corresponding enhancement of the cellulolytic activity of the preparations (Wood et al., 1988). The same has been observed upon addition of cellobiohydrolase I from *T. reesei* to the endoglucanase from a low-value cellulase preparation of *Aspergillus niger* (Lee et al., 1988).

(ii) All the full-value cellulase systems studied possess endoglucanases that adsorb tightly on cellulose (Rabinovich et al., 1981, 1983; Klyosov et al., 1986). Moreover, the more catalytically active the cellulase preparations are in relation to the solubilization of crystalline cellulose, the more tightly adsorbed are the endoglucanases they contain (Figure 2). These and other similar data (see below) formed a basis for the rule: The better the adsorption, the better the catalysis (Klyosov et al., 1986). This rule is elaborated below, in the section on the adsorption of cellulases on cellulose.

(iii) Recent experimental data on the reactivity of purified endoglucanases from a number of cellulase systems have led to a rather unexpected conclusion: the ability of individual endoglucanases to split crystalline cellulose turns out to be directly associated with their ability to form *glucose* as a soluble product of the hydrolysis. In other words, the more random acting the endoglucanase, the less active they are in relation to the hydrolysis of crystalline cellulose, and the less glucose (compared with that of cellobiose) they form as the products of the hydrolysis of crystalline cellulose (Figure 3).

Two conclusions follow from these data (Figure 3): (i) the ability to form glucose might be more important for the activity of an individual endoglucanase toward crystalline cellulose than the ability to adsorb tightly onto cellulose; (ii) the rule the better the adsorption, the better the catalysis is not absolute. It may be a strong rule either for unresolved cellulase systems or for individual endoglucanases from *the same* microorganism (see Figure 7).

Thus, if an endoglucanase has a low reactivity toward crystalline cellulose, it forms, almost exclusively, cellobiose as the reaction product. On the contrary, a more reactive endoglucanase produces more glucose. The latter is apparently formed as a result of transglucosylation catalyzed by the endoglucanase (see below). Transglucosylation reactions, therefore, might play an important role in making full-value cellulase systems. Indeed, a non-cellobiase pathway for glucose formation plays an important role in the action of full-value cellulase systems (Klyosov & Rabinovich, 1980).

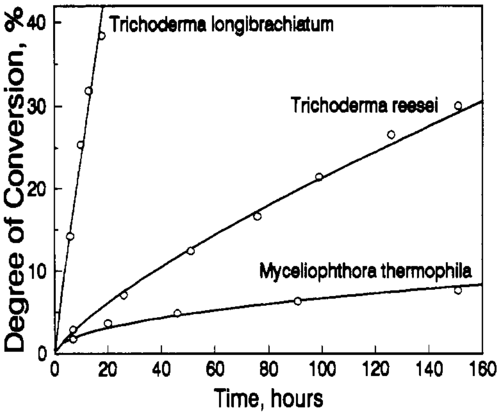

FIGURE 3: Hydrolysis of crystalline cellulose by the most tightly adsorbed endoglucanases purified to homogeneity from *T. longibrachiatum*, *T. reesei*, and *M. thermophila* (in the presence of an excess of cellobiase; that is, all of the product formed is glucose). The ordinate shows the degree of conversion of cellulose into glucose. The respective partition coefficients were 0.55, 2.8, and 2.7 L/g, the degree of "orderliness" of their action on soluble CM-cellulose (that is, the ratio of the saccharifying activity to the liquifying one) was 6.2, 3.5, and 1.3, and the ratio of glucose to cellobiose as the products of the hydrolysis of crystalline cellulose (without added cellobiase) was 1:0.67, 1:1, and 1:8, with catalytic constants of 0.67, 0.51, and 0.013 s^{-1}, respectively. In relation to the viscometric analysis of the hydrolysis of soluble CM-cellulose, the order of the enzymes was the reverse, with catalytic constants of 19, 40–60, and 140 s^{-1}, respectively (Klyosov et al., 1987).

BREAKDOWN OF *p*-NITROPHENYL GLUCOSIDE BY ENDOGLUCANASE AND MECHANISM OF GLUCOSE FORMATION FROM POLYMERIC CELLULOSE: TRANSGLUCOSYLATION

For a long time it was regarded as an established principle that endoglucanases did not split short derivatives of glucose, such as cellobiose or *p*-nitrophenyl β-glucoside (*p*-NPG). Endoglucanases indeed have been inactive on these compounds, indicated by their specificity and nomenclature to be enzymes of the endo-wise type. It turned out, however, that *in the presence of polymeric cellulose* (CM-cellulose as well as amorphous or crystalline cellulose) a highly purified endoglucanase from *T. viride* rapidly cleaved *p*-nitrophenyl glucoside (that is, a rapid accumulation of *p*-nitrophenol occurred). The introduction of insoluble cellulose (microcrystalline or amorphous) into the reaction mixture led to an increase in the rate of *p*-NPG breakdown by endoglucanase of 200–400 times. The introduction of soluble CM-cellulose led to an even higher acceleration of the reaction rate. This results in a rate of conversion of *p*-NPG by the endoglucanase comparable to the rate of hydrolysis of specific polysaccharide substrates by this enzyme (Kraeva et al., 1986).

Such an unexpected change in the established specificity of the endoglucanase seems to be unexplainable at first sight. However, analysis of the composition of the residual insoluble substrate clarifies the picture. As was revealed, during the course of the enzymatic reaction, incorporation of the *p*-nitrophenyl residue into cellulose occurred (Kraeva et al., 1986). The simplest explanation of the phenomenon may be that the breakdown of *p*-NPG by endoglucanase is via a transglucosylation mechanism with intermediate formation of insoluble *p*-nitrophenyl cellooligosaccharides (covalently bound to the insoluble cellulose matrix), from which the enzyme splits *p*-nitrophenol.

Developing this hypothesis further, it might be supposed that some endoglucanases can convert *cellobiose* to glucose via the transglucosylation mechanism *in the presence of cellulose.* If this is the case, it becomes clear why some full-value cellulase systems, having little or practically no cellobiase, produce so much glucose (the typical case, cellulases from *T. reesei*).

ADSORPTION OF CELLULASES ON CELLULOSE SURFACE: PHENOMENOLOGY OF THE ADSORPTION, TIGHTNESS OF THE ADSORPTION, AND ROLE OF THE ADSORPTION IN CATALYSIS

Adsorption of cellulases on cellulose provides not only a physical contact between the enzyme and the substrate. Adsorption in many cases plays an important role in the efficiency of the enzymatic hydrolysis of cellulose, crystalline cellulose in the first turn (Figures 2 and 5–7). As mentioned in a previous section, the rate of the enzymatic hydrolysis of crystalline cellulose is often determined by the rule: the better the adsorption, the better the catalysis (Klyosov et al., 1986; Klyosov, 1988).

The efficiency of cellulase adsorption on the surface of cellulose can be characterized by the partition coefficient (K_p) of the enzyme between the substrate surface and the water phase. K_p is numerically equal to the ratio of the quantity of the enzyme adsorbed by a unit of mass, usually 1 g (or surface, usually 1 m^2), of the adsorbent to the equilibrium concentration of the enzyme in the bulk solution under conditions of linearity of the adsorption isotherm (Rabinovitch et al., 1981, 1983; Klyosov et al., 1986, 1987). The adsorption equilibrium is usually reached in 1–3 min (Klyosov et al., 1982). It has been found that the adsorption ability of endoglucanases from various sources to crystalline cellulose varies significantly, in the range of more than 1000 times: from the very weakly adsorbed endoglucanase from *Aspergillus foetidus* (K_p 0.0035 L/g) up to the tightly adsorbed endoglucanase from *Clostridium thermocellum* (L_p 5.5 L/g).

The discovery of the binding domain in some cellulolytic enzymes explains in part the difference in the adsorption ability of cellulases. But the question remains: why do binding domains of different cellulases differ in their affinity for cellulose? One reason might be the carbohydrate moiety of the domains, for the carbohydrate part of cellulases has been shown to be important in adsorption of the enzymes onto cellulose (Klyosov et al., 1987; Chernoglazov et al., 1988; Bhat et al., 1989). Then, according to recent data (Tikhomirov et al., 1987) there is, in some cases, a certain correlation between the hydrophobicity of endoglucanases and their ability to adsorb onto cellulose.

Data on the adsorption ability of endoglucanses are listed for 26 cellulase preparations, 10 of which are highly purified (Klyosov, 1988). Tightly bound endoglucanases are formed by some fungi (*Trichoderma*, *Myceliophthora*, *Aspergillus*), bacteria (*Clostridium*), and actinomyces (*Actinomyces diastaticus*). Numerous data show that the adsorption ability of endoglucanses plays the principal role in the hydrolysis of *amorphous* and *crystalline* cellulose. In order to illustrate this, we shall cite a few experimental data. It is worthwhile noting that the adsorption capacity of endoglucanases does not depend on the degree of crystallinity of cellulose (Figure 4). Considering the hydrolysis mechanism, it should be emphasized that the higher the crystallinity of cellulose, the slower it is to hydrolyze by weakly adsorbed enzymes. On the contrary, it is hydrolyzed rather well by tightly adsorbed enzymes (at the same surface concentration of the enzymes). These data are shown in Figure 1. It is remarkable that the intersection point with the vertical axis in Figure 1 (where the degree of crystallinity is equal to zero or, to be more correct, where the term crystallinity loses its sense) corresponds to the hydrolysis of CM-cellulose, as shown experimentally. Thus, this plot (Figure 1) accommodates data for all the three types of cellulose substrate, i.e., soluble, amorphous, and crystalline cellulose, and allows one to follow the change of the reactivity of cellulases along all the three types of cellulose.

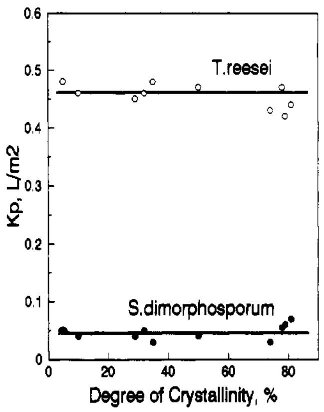

FIGURE 4: Effect of degree of crystallinity of cellulose on adsorption of endoglucanases (crude preparations) from two microbial sources. To equalize specific surfaces of all the 10 cellulose samples, the partition coefficients have been measured as per a surface unit (Klyosov et al., 1982).

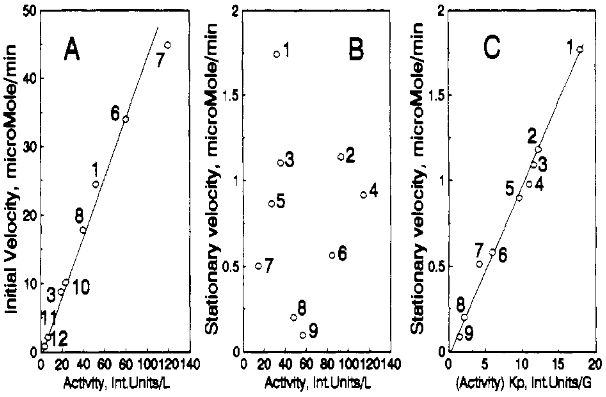

FIGURE 5: Hydrolysis of amorphous (panel A) and crystalline (panels B and C) cellulose by cellulase complexes with an excess of added purified cellobiase. (Panel A) The velocity of the hydrolysis of amorphous cellulose is proportional to the endoglucanase activity in the reaction system. (Panel B) The velocity of the hydrolysis of crystalline cellulose shows no correlation with the endoglucanase activity; note that some cellulases with *low* endoglucanase activity can efficiently degrade crystalline cellulose (e.g., cellulases from *Trichoderma*) whereas some cellulases with rather *high* endoglucanase activity degrade it much slower (e.g., cellulases from *M. verrucaria* or *S. dimorphosporum*). (Panel C) The velocity of the hydrolysis of crystalline cellulose is proportional to the *fraction* of endoglucanase activity determined by the *adsorption ability* of the enzyme (see the abscissa values). Source of the complexes: (1) *T. viride*, (2) *A. terreus*, (3) *T. longibrachiatum*, (4) *M. verrucaria*, (5) *T. viride* (Onozuka R-10), (6) *G. candidum*, (7) *S. dimorphosporum*, (8) *A. niger*, (9) *T. viride* (Boehringer Mannheim), (10) *T. lignorum*, (11) *A. foetidus*, and (12) *Thermomonospora* sp. (Klyosov et al., 1986c).

The principal differences in the hydrolysis of amorphous and crystalline cellulose by cellulases having different adsorption abilities can be seen in Figure 5. The data show that, for efficient hydrolysis of *amorphous* cellulose, only the quantity (that is, *activity*) of a cellulase preparation is important. On the contrary, for the hydrolysis of *crystalline* cellulose not only the quantity of cellulases is important but mainly their *quality*, that is, the ability of endoglucanases to be strongly adsorbed on the surfaces (Rabinovich et al., 1981, 1983; Klyosov et al., 1982, 1983, 1986; Klyosov, 1988).

Another illustration of how important the adsorption of endoglucanases is for the hydrolysis of *crystalline* cellulose is

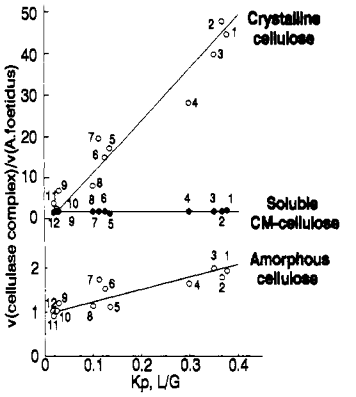

FIGURE 6: Effect of K_p on the hydrolysis of soluble, amorphous, and crystalline cellulose by cellulase complexes. K_p, adsorption ability of endoglucanases (expressed as the partition coefficient). The velocities of the hydrolysis are shown as the ratio to those by cellulase from *A. foetidus*. The hydrolysis was performed with an excess of purified cellobiase. Cellulase preparations are listed in Figure 2 (Klyosov et al., 1982).

given in Figure 6. Here for 12 cellulase preparations from various microbial sources it is shown how the hydrolysis of soluble, amorphous, and crystalline cellulose and how their respective reactivity depend on their partition coefficients (K_p). Figure 6 shows that an increase in the adsorption capability of endoglucanases of about 20-fold (K_p from 0.02 up to 0.37 L/g) does not change the rate of hydrolysis of CM-cellulose; the rate of hydrolysis of amorphous cellulose is increased about 2-fold, whereas the rate of hydrolysis of crystalline cellulose is increased in about 50 times (Klyosov et al., 1982). It should be noted that this increase in the hydrolysis rate is proportional to the adsorption capability of cellulases. If a cellulase binds fairly tightly with cellulose, the reactivity of the crystalline substrate to enzymatic hydrolysis increases so much that it reaches nearly 30% of the reactivity of amorphous cellulose (Klyosov et al., 1982).

All these examples are related to the action of endoglucanases in a mixture of other enzymes of *cellulase complexes*. In order to demonstrate the validity of the rule "the better the adsorption, the better the catalysis" for the action of individual endoglucanases, the respective data are shown for six highly purified multiple forms of endoglucanases from *T. reesei* (Figure 7). Indeed, the increase of the adsorption capability of the purified enzymes goes in parallel with their reactivity toward crystalline cellulose (Klyosov et al., 1987).

Further studies have shown, however, that this rule has some limitations. Some exceptions from the rule "the better the adsorption, the better the catalysis" are described above (see Figure 3) and are related to some individual endoglucanases from a few *different* fungi. A possible explanation for the exceptions is that endoglucanases from various sources can differ significantly not only in adsorption capability but also in a number of other important properties, e.g., the ability to catalyze transglycosylation reactions with the formation of glucose. The latter property might play a role in the enzymatic degradation of crystalline cellulose no less significant the adsorption ability of the enzymes on cellulose.

So why are tightly adsorbed endoglucanases significantly more reactive in relation to the hydrolysis of crystalline cellulose than weakly adsorbed enzymes? Why are the differences in the efficiency of the action of tightly and weakly adsorbed endoglucanases significantly less expressed in the hydrolysis of amorphous cellulose? These are key questions for the contemporary biochemistry and enzymology of cellulose hydrolysis. The answer to them is apparently bound to the mechanochemical action of cellulases to crystalline cellulose and their behavior as surfactants as described in the next section.

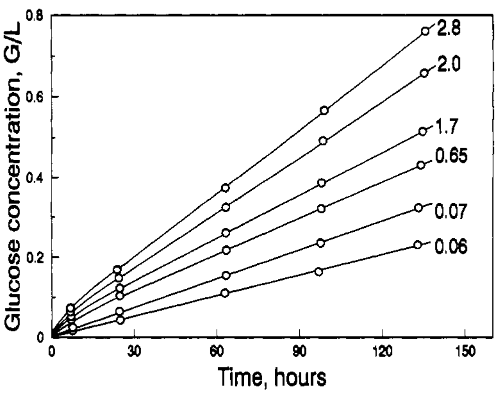

FIGURE 7: Hydrolysis of crystalline cellulose (1 g/L) to glucose by highly purified multiple forms of endoglucanase from *T. reesei*. Figures at the curves indicate the adsorption ability of the enzymes (expressed as the partition coefficients). The hydrolysis was performed with an excess of cellobiase (Klyosov et al., 1987).

MECHANOCHEMICAL ACTION OF CELLULASE ON CELLULOSE: DISORDERING, DEFIBRILLATION, AND DISPERSION

The phenomenon of defibrillation, or dispersion of cellulose, at initial steps of hydrolysis under the action of cellulases has been known since the beginning of the 1960s and was described in detail in the work of Halliwell and King (Halliwell, 1966; King, 1966; Halliwell & Riaz, 1970). More recently, it was shown that the first step in the enzymatic degradation of the microfibrils of cellulose was their stratification along the longitudinal axis leading to the formation of even more thin subfibrils (White & Brown, 1981; Chanzy & Henrissat, 1983; Chanzy et al., 1983). Though, the opinions about which enzyme of the cellulase system was the key one in the dispersion (or defibrillation) of crystalline cellulose were divided. According to the American authors (White & Brown, 1981) this enzyme was endoglucanase; according to the French authors (Chanzy et al., 1983) it was cellobiohydrolase I.

Recently, convincing data has been obtained (Rabinovich, 1988) that both endoglucanase and cellobiohydrolase can bring about the dispersion of cellulose and that the dispersion can result from hydrolytic and mechanical action on cellulose. Hydrolytic dispersion takes the form of enzymatic cleavage of cellulose macromolecules. The mechanical dispersion is induced by the adsorption of (cellulolytic) enzymes to cellulose defects (disturbances of the crystalline structure of cellulose, e.g., microcracks) followed by their penetration into the interfibrillar space (Rabinovich et al., 1982). This in turn concentrates the enzyme in the defects (as a result of a larger contact of binding sites of the enzyme with the cellulose surface), resulting in an increase in the mechanical pressure on the walls of pores, cavities, and microcracks of cellulose (Klyosov & Rabinovich, 1980). Water in the defects in turn penetrates further and further inside the capillar spaces breaking hydrogen bonds between the cellulose molecules, forcing apart and solvating the microcrystallites. In turn, the adsorbed cellulases prevent the newly formed surfaces from sticking back together. This is a tentative mechanism for the enzymatic dispersion not only of microcrystalline cellulose but also of native cellulose with a high degree of polymerization, e.g., cotton (Rabinovich, 1988).

This process can be easily visualized by means of a simple experiment (Rabinovich, 1988) in which microcrystalline

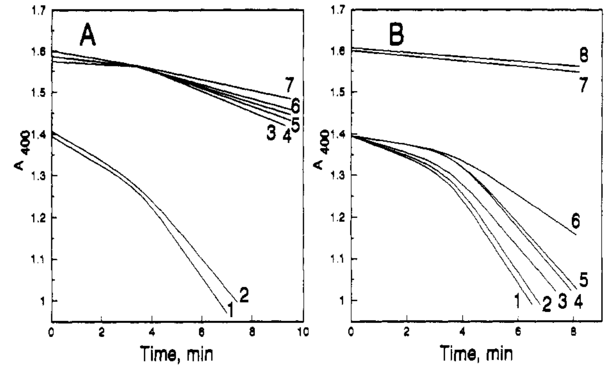

FIGURE 8: Effect of cellulase preparations (panel A) and other proteins (panel B) on the coagulation and precipitation of a sonicated microcrystalline cellulose suspension (0.4 g/L). The velocity of the precipitation of the suspension was measured by the optical absorption at 400 nm directly in a spectrophotometer cuvette. (Panel A) Cellulases added to the initial suspension: (1) none, (2) *T. viride* (Boehringer Mannheim; see Figure 2), (3) *T. viride* (Serva), (4) *A. terreus*, (5) *T. reesei* (Novo), (6) a purified cellulase preparation from *T. viride*, and (7) *T. viride* (Meicellase). (Panel B) (1 and 4) Initial suspension (the extreme positions); in the presence of (2) glycerol-3-phosphate dehydrogenase, (3) peroxidase, and (5) hexokinase (all at a concentration of 0.040 mg/mL; (6 and 8) cellobiohydrolase from *T. reesei* (0.001 and 0.005 mg/mL, respectively); (7) tightly adsorbed endoglucanase from *T. reesei* (0.005 mg/mL) (Rabinovich, 1988).

cellulose (Avicel) is placed as an aqueous suspension in an ultrasonic field for a few minutes and then left to a spontaneously precipitate (coagulation), which is recorded spectrophotometrically. During the first 3–4 min after sonication, the suspension is relatively stable, and its optical density does not change. That is, the coagulation of small nonaggregated particles of cellulose proceeds slowly. Then, a period of rapid coagulation and precipitation of flakes occurs, and the respective optical density of the suspension decreases sharply (Figure 8A, curve 1). However, if *tightly adsorbed cellulases* are added to the suspension right after sonication, then even with a very small amount of enzymes added (0.02–0.04 mg/mL) the suspension is stabilized, and the velocity at which the particle precipitates is significantly decrease (Figure 8A, curves 3–7; Figure 8B, curves 7 and 8). *Weakly adsorbed cellulases* do not appreciably affect the sedimentation rate (Figure 8A, curve 2), as well as other non-cellulase proteins which have been added to the suspension (bovine serum albumin, ovalbumin, human γ-globulin, trypsin, α-chymotrypsin, lysozyme, horseradish peroxidase, β-glucosidase, glucose oxidase, hexokinase, horse liver alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase; some of them are shown in Figure 8B, curves 2–5).

Repeated shaking of the cellulose suspension with the tightly adsorbed enzymes stabilized it further, the maximal effect of the stabilization developing over 30–40 min. Moreover, the optical density of the suspension after addition of small amounts of the tightly adsorbed enzyme followed by shaking is higher than the initial value of OD (in the control) as a result of smaller particle formation (Figure 8). This effect was certainly brought about by the mechanical dispersion of cellulose under the action of cellulases because the time of contact of cellulose with the enzymes was too short to expect any significant hydrolytic dispersion (Rabinovich, 1988).

Thus, tightly adsorbed cellulases, both endoglucanases and cellobiohydrolases, but not loosely adsorbed cellulases and other proteins, bring about mechanical dispersion of cellulose particles and prevent their adhesion. These effects certainly play an important role in the enzymatic degradation of insoluble (native and microcrystalline) cellulose. Apparently, for amorphous cellulose, all the surface is easily accessible to enzymatic attack; therefore, the penetration of enzymes to inner areas of the substrate does not necessarily need mechanical dispersion of cellulose with the help of tightly adsorbed cellulases to prevent stabilized microfibrils from sticking together. Because of a high degree of hydration and the mobility of cellulose macromolecules, the hydrolysis of amorphous cellulose under the action of weakly adsorbed cellulases is quite possible. In the case of crystalline cellulose, though, as can be seen from the above data, the presence of tightly adsorbed cellulases is necessary.

BEHAVIOR OF ADSORBED CELLULASES ON THE CELLULOSE SURFACE

What is the consequence of events that take place on the cellulose surface after a successful attack by the adsorbed enzyme on the glucosidic bond of an insoluble cellulosic substrate? Two major variants can be visualized: first, the attack is followed by the desorption of the enzyme into the bulk solution with subsequent readsorption onto another part of the substrate, or second, the enzyme moves along the cellulose surface performing a series of successive attacks without desorbing from the surface. In the latter case the above question might be posed as follows: how many catalytic acts (on average) does the adsorbed enzyme make before it desorbs from the cellulose surface?

A novel theoretical and the experimental approach (Rabinovich et al., 1984, 1985a,c) can help to answer this question. It also allows one to obtain data on the adsorption capacity of cellulosic materials in relation to cellulases and on the velocity of desorption of cellulases from the cellulosic materials into the bulk solution. It appears that cellulolytic enzymes perform hundreds and thousands of catalytic acts without leaving the cellulose surface. At the same time the transfer of the enzymes between cellulose particles occurs much faster that the complete degradation of these particles. Finally, this approach allows one to obtain data on the character and velocity of the "erosion" (or, on the contrary, of the "smoothing") of the cellulose surface in the course of its enzymatic attack (Rabinovich et al., 1985a).

The approach is based on the use of (a) cellulose-containing adsorbed enzymes and (b) a standard dyed insoluble cellulose, added into the same reaction system. Some time after both preparations of cellulose are mixed, the dye appears in the solution as the result of the redistribution of the enzyme between the surfaces of the nondyed (under study) and dyed (standard) cellulose. Kinetic analysis of the formation of the dyed product in the system has shown (Rabinovich et al., 1985c) that the time lag corresponds to the period in which the enzyme remains bound to the substrate particle (on average) upon which the enzyme was adsorbed; the stationary phase of the curve corresponds to the quantity of enzyme transferred from the nondyed cellulose onto the dyed one before the new adsorption equilibrium is attained in the system.

Experiments with various cellulases and various cellulose sampels have shown that the average lifetimes of cellulases on the surface of microcrystalline cellulose were 25–35 min; on amorphous cellulose (regenerated from phosphoric acid) they were 60–75 min. As the turnover number (or the catalytic constant) for endoglucanases from various microbial sources toward crystalline cellulose usually varies from 0.01 to 0.7 s^{-1} and that toward amorphous cellulose varies from 1.0 to 5.6 s^{-1}, one can easily calculate that cellulases perform dozens and hundreds of catalytic acts (hydrolyzing crystalline cellulose) or even thousands of acts (hydrolyzing amorphous cellulose) without leaving the surface of a cellulose substrate.

SYNERGISM BETWEEN COMPONENTS OF THE CELLULASE SYSTEM: MOLECULAR MECHANISMS FOR THE ENZYMATIC HYDROLYSIS

Synergism is a phenomenon resulting in a mutual increase of the efficiency of action of two or more components of a system when they act together in comparison with their additive action when they act separately. For cellulose hydrolysis with enzymes of the cellulase system synergism results in that the velocity of formation of soluble products and the degree of conversion of cellulose under simultaneous action of several components of the cellulase system exceed the sum of velocities of formation of the products when the components act separately.

Synergism is one of the most remarkable features of cellulase systems, and it differentiates, as a rule, complete cellulase systems from noncomplete ones. Synergism in cellulase systems was described first over one-quarter of a century ago (Mandels & Reese, 1964), and since that time, its possible molecular mechanisms have become the subject of many discussions. First, it is not clear why the addition of *cellobiohydrolase* enhances the *action of endoglucanase* on insoluble (particularly crystalline) cellulose. Second,it is not clear why far from all endoglucanases and cellobiohydrolases exhibit synergism at the joint action, which should be the case in "kinetic interaction" of the enzymes. Indeed, the cross-synergism between endoglucanase from one microbial source and cellobiohydrolase from another one, or that between cellobiohydrolase and different endoglucanases from the same source, is not often observed (Wood & McCrae, 1986b; Wood et al., 1988, 1989; Gow & Wood, 1988). Third, it is not clear how the simple interpretation of the synergism can explain the exo-exo synergism between *two cellobiohydrolases* found by a few authors on the hydrolysis of insoluble cellulose (Fagerstam & Pettersson, 1980; Henrissat et al., 1985; Wood & McCrae, 1986b; Woodward et al., 1988). Finally, another kind of synergism was found, that is, between *two endoglucanases*, on the hydrolysis of crystalline cellulose (Rabinovich et al., 1986b), which cannot be explained in the framework of the simple "consecutive" action of endo and exo enzymes either.

Let us consider the last example in more detail for it apparently gives a key to the explanation of the puzzling phenomena of synergism. The synergism between two endoglucanases is observed for the enzymes, one of which is adsorbed *tightly* and the other *weakly* (Rabinovich et al., 1986b). The adsorption constants, that is, the partition constants for these endoglucanases (from *T. viride*) between the cellulose surface and the water phase, differed by 10 times and were equal to 1.3 ± 0.1 and 0.15 ± 0.02 L/g. The joint action of the two enzymes has led to a much higher degree of hydrolysis of microcrystalline cellulose than the separate actions of each of the individual enzymes. Moreover, for the degree of conversion of the substrate (10, 20, 30, 35, and 40%), the extent of the synergism (measured in relation to the velocity of glucose formation in the presence of an excess of cellobiase) was equal to 1.3, 1.5, 2.0, 2.3, and 2.5, respectively (Rabinovich et al., 1986b).

Under the action of a mixture of the tightly and loosely adsorbed endoglucanases the conversion degree of microcrystalline cellulose was higher than 75%, whereas the loosely adsorbed endoglucanase has practically ceased its action at a degree of conversion of 30–40% (even when more enzyme was added into the reaction system). The study showed (Rabinovich et al., 1986b) that the sites for the attack of the substrate by loosely and tightly adsorbed endoglucanases were different, and the addition of the *loosely* adsorbed endoglucanase to the cellulose previously hydrolyzed with the *tightly* adsorbed enzyme lead to a significant increase in the velocity of the hydrolysis as well as in the degree of conversion of the substrate even at the high (more than 60%) degrees of conversion of the microcrystalline cellulose. Moreover, the experiments have shown that the rate of hydrolysis of the substrate under joint action of the loosely and tightly adsorbed cellulases was 6 to 15–20 times higher than that under action of the tightly adsorbed cellulases only (Rabinovich et al., 1986b).

All of these data have allowed us to put forward the following statement: the efficiency of the degradation of crystalline cellulose is enhanced when the enzyme system contains cellulases with *both high and low* affinity to cellulose, because they show synergism when acting simultaneously. The molecular mechanisms of the exo-endo and endo-endo types of synergism are principally different. It seems that tightly bound enzyme (with K_p of 1–5 L/g) binds to cellulose at sites where the crystalline structure of the substrate is disturbed and induces a dispersion (defibrillation) of the crystallites as a result of the corresponding mechanochemical effects (see above). These tightly adsorbed enzymes can then penetrate into intercrystalline regions and open new sites for the action of weakly adsorbed enzymes (with K of 0.02–0.04 L/g). The latter in turn act rapidly only toward disordered (amorphous) regions of cellulose, situated at peripheral parts of microcrystallites. They could also become accessible as a result of the dispersion of cellulose crystallites. This apparently is the main reason for the synergism between tightly and weakly adsorbed cellulases—not only between endoglucanases but also between cellobiohydrolases as well as between their combinations.

The above can explain most (if not all) of the puzzling features of endo-exo, exo-exo, and endo-endo synergisms described in this section. It now becomes clearer as to why the cross-synergism between endoglucanases and cellobiohydrolases from different sources (Gow & Wood, 1988) has been found only for the enzymes from *complete* (*full-value*) cellulase systems but was not observed for any combinations of cellobiohydrolases from complete cellulase systems and endoglucanases from *noncomplete* (*low-value*) cellulases (of the fungi *M. verrucaria*, *S. atra*, and *M. echinata* or the bacteria *R. albus*, *R. flavefaciens*, and *B. succinogenes*). The apparent reason is that all known *complete* cellulase systems contain *tightly* adsorbed endoglucanases (see above) and that all known endoglucanases from the above listed fungal and bacterial sources of *noncomplete* cellulase systems are *weakly* adsorbed on cellulose (Klyosov, 1988). Gow and Wood (1988) came closest to the explanation of the phenomenon when indicating that synergism between fungal cellobiohydrolase and endoglucanase originating in another microorganism occurs only in those situations where the endoglucanase has been isolated from a cellulase preparation that also contained a cellobiohydrolase; i.e., the cellulase preparation from which it was isolated was a "true" cellulase.

In terms of molecular mechanisms and from the viewpoint of the approach developed here, it seems that the synergism occurs only in those situations where the endoglucanase is able to tightly adsorb onto cellulose. It takes place indeed for all major forms of endoglucanases from true cellulase systems (Klyosov, 1988). This conclusion is in a line with results of a recent work (Lee et al., 1988) in which no synergism was found in a joint action of tightly adsorbed cellobiohydrolase I from *T. reesei* and either of two *weakly adsorbed* endoglucanases from *A. niger*. Recent work by Wood and coworkers (Bhat et al., 1989; Wood et al., 1989) has demonstrated that a mixture of cellobiohydrolases I and II from *P. pinophilum* showed a significant synergism only with *tightly* adsorbed endoglucanses III and V from the same source and not with *weakly* adsorbed endoglucanases I, II, and IV. These serve as convincing evidence of the importance of the tight adsorption of endoglucanases for development of synergism with cellobiohydrolases.

In the light of this concept it is not surprising that endoglucanase from the bacterium *C. thermocellum* shows a cross-synergism in a joint action with cellobiohydrolase from *T. koningii* (Gow & Wood, 1988) because the bacterial endoglucanase is very tightly adsorbed on cellulose (Klyosov, 1988).

The existence of the synergism between two cellobiohydrolases may now be explained. If one of them is able to be tightly adsorbed on cellulose, it may lead to the mechanochemical effects and disperse crystalline cellulose, to give thereby a possibility for the second cellobiohydrolase to attack newly formed surfaces and/or exposed amorphous parts of cellulose. The experimental verification of this suggestion has shown that cellobiohydrolase I from *T. reesei* indeed binds more tightly to crystalline cellulose (as well as to cellophane and viscose) than does cellobiohydrolase II. This was shown with the help of a new method named "affel" (*aff*inity + *el*ectrophoresis), which combines the affinity binding of cellulases onto cellulose with the subsequent isoelectric focusing of proteins tightly adsorbed on the carrier (Rabinovich et al., 1985b). The cellulose particles with adsorbed cellulases (directly from the culture liquid of *T. reesei*) after thorough rinsing with water were introduced into polyacrylamide gel. Then proteins were subjected to electrodesorption and isoelectric focusing. It was observed that cellobiohydrolase I was completely transferred from the culture liquid onto the cellulose surface, whereas the adsorption of cellobiohydrolase II was much less.

At last, on the basis of the modes of action of tightly and weakly adsorbed cellulases, it becomes clearer why not all cellobiohydrolases and endoglucanases show synergism when acting together in a mixture. For example, according to Wood et al. (1988) only one of two cellobiohydrolases from *P. pinophilum* showed synergism with cellobiohydrolase of *T. koningii* and cellobiohydrolase of *F. solani*. Then, only two of four endoglucanases of *T. koningii* show synergism (Wood & McCrae, 1978) with cellobiohydrolase toward crystalline cellulose. Wood and McCrae have suggested that it could be explained either by the existence of specific associates between endoglucanase and cellobiohydrolase on the surface of cellulose (it is not clear though why some pairs of cellulases do form associates while others do not) or by the existence of two different stereospecific cellobiohydrolases concerned with the hydrolysis of the two different configurations of nonreducing end groups that would be expected to exist in the cellulose crystallite (Wood & McCrae, 1986b; Wood et al., 1988, 1989). It seems that the last suggestion can neither be proved nor be denied, and therefore, it can hardly be considered here. The explanation, though, may turn out to be much simpler and, more importantly, be verified experimentally—if the synergism is caused by one of the components being adsorbed tightly on cellulose. If both the components are adsorbed weakly on cellulose, synergism would not be observed, or observed in a much less extent. This might possibly explain recent observations (Wood et al., 1989) that there was little or no synergistic activity either between purified cellobiohydrolases I and II or between the individual cellobiohydrolases and the endoglucanases of *P. pinophilum*.

In conclusion, considerations concerning tightly and loosely adsorbed cellulolytic enzymes seemed to be rather fruitful for the explanation of both the puzzling features of synergism in the action of cellulolytic components toward insoluble cellulose and the mechanism of the enzymatic hydrolysis of crystalline cellulose.

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Accelerated Publications

A Novel Minimum Ribozyme with Oxidoreduction Activity

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ABSTRACT: A nucleoside catalyzing the oxidoreduction of NADH and $K_3Fe(CN)_6$ was isolated from *Torula* yeast RNA and also obtained in 0.05% yield by a series of steps: SDS-phenol extraction, nuclease P_1 digestion, alkaline phosphatase digestion, anion exchange chromatography, and HPLC on an ODS column. Its chemical structure was clearly determined at 5-hydroxycytidine, from the results of FAB-MS and ^{1}H and ^{13}C NMR spectroscopies. The mass spectra, chromatographic behavior, UV spectra, and NMR spectra of this nucleoside from natural and synthetic sources were identical. This is the first report of an RNA catalyst having catalytic activity except for the cleavage and ligation of phosphodiester bonds of RNA. That an RNA has oxidoreduction activity indicates new possibilities for RNAs as "living molecules". 5-Hydroxycytidine may be a vestige of RNAs that formerly possessed metabolizing ability.

Until quite recently, RNA was considered a passive carrier of information stored in DNA (Crick, 1970). In the last few years, it has been found to have self-catalytic activity for splicing (Cech, 1981) and cleavage (Stark et al., 1978) of RNA, thus causing a change of image. Recent new results strongly support the possibility that RNA, not DNA, is the first carrier of information and is capable of replicating itself in the absence of protein enzymes (Gilbert, 1986; Sharp, 1985). Both these features may have been possessed by the same molecule in the early stages of biological evolution. Such RNAs are presently regarded as the legacy of primordial RNA (Pace & Marsh, 1985; Joyce, 1989).

Known catalytic RNAs (ribozymes) (Cech & Bass, 1986; Cech, 1987) can be categorized as group I RNA IVS[1] (Cech,

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[1] Abbreviations: IVS, intervening sequence; RNP, ribonucleoprotein; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; h^{5}C, 5-hydroxycytidine; 2D NMR, two-dimensional nuclear magnetic resonance; k_{cat}, catalytic rate constant; K_m, Michaelis constant; 2,6-DCIP, 2,6-dichlorophenolindophenol; β-NMNH, β-nicotinamide mononucleotide, reduced form; β-NHypDH, β-nicotinamide hypoxanthine dinucleotide, reduced form; 3-AcPyADH, 3-acetylpyridine adenine dinucleotide, reduced form.

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