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Kinetics and Thioredoxin Specificity of Thiol Modulation of the Chloroplast H^+-ATPase*

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The kinetics of thiol modulation of the chloroplast H^+-ATPase (CF_0CF_1) *in membrana* were analyzed by employing thioredoxins that were kept reduced by 0.1 mM dithiothreitol. The kinetics of thiol modulation depend on the extent of the proton gradient. The process is an exponential function of the thioredoxin concentration and reaction time and can be described by an irreversible second order reaction. The results indicate that the formation of the complex between thioredoxin and CF_0CF_1 is slow compared with the subsequent reduction step. Furthermore we have compared the efficiencies of the *Escherichia coli* thioredoxin Trx and the two chloroplast thioredoxins Tr-m and Tr-f. The second order rate constants are 0.057 (Tr-f), 0.024 (Trx), and 0.010 s^{-1} μM^{-1} (Tr-m) suggesting that Tr-f rather than Tr-m is the physiological reductant for the chloroplast ATPase. The often employed artificial reductant dithiothreitol exhibits a second order rate constant in thiol modulation of $1.02 \cdot 10^{-6}$ s^{-1} μM^{-1}.

The H^+-translocating ATPase of chloroplasts (CF_0CF_1,[1] chloroplast ATP synthase) is a latent enzyme. Its physiological activation requires a transmembrane electrochemical proton potential difference (1–4). Hence, the proton gradient in addition to its role as the driving force of phosphorylation is a factor that controls CF_0CF_1 activity. The obvious physiological meaning of this control mechanism is the suppression of unproductive ATP hydrolysis under conditions that would energetically allow this reaction, *i.e.* at low proton gradients (low light or dark) and high phosphate potentials.

A superimposed regulatory device is the so-called thiol modulation of CF_0CF_1. The structural basis for thiol modulation is a sequence motif of nine amino acids comprising two cysteines in the γ subunit of CF_1 (5). This segment is present in higher plants (6) and green algae (7) but not in cyanobacteria (8–10) or in diatoms (11) suggesting that thiol modulation is an acquisition of the chlorophyll $a + b$ plants only. In the demodulated (oxidized) state the two cysteines form a disulfide bond whereas the modulated state is obtained by reduction of this disulfide bridge. *In vitro* reduction can be achieved by dithiothreitol or other dithiols, but the natural reductant is a reduced thioredoxin. In chloroplasts at least two different thioredoxins occur, thioredoxin-m (Tr-m) and thioredoxin-f (Tr-f) (12). The former is thought to be involved in light/dark regulation of the chloroplast NADP-specific malate dehydrogenase, and the latter is responsible for the light/dark regulation of fructose bisphosphatase and other Calvin cycle enzymes (13, 14). The thioredoxins are reduced via ferredoxin and ferredoxin-thioredoxin reductase (15) by electrons from the photosynthetic electron transport chain. In most of the experiments carried out so far, however, thiol modulation of CF_0CF_1 was conducted with the artificial reductant dithiothreitol, and in a few studies *Escherichia coli* thioredoxin (Trx) was used (16, 17). Little information is known about the action of the naturally occurring chloroplast thioredoxins on CF_0CF_1 (18–20).

Thiol modulation requires illumination of the chloroplasts to allow reduction of the disulfide bridge. Apparently, the regulatory segment of the γ subunit, which is hidden in the dark, becomes accessible as a consequence of $\Delta\mu H^+$-induced CF_0CF_1 activation (16, 21). Decay of the proton gradient in the dark leads to deactivation of the ATP synthase. The most significant difference between the reduced and oxidized active states concerns the velocity of deactivation. While the oxidized form is immediately deactivated upon relaxation of the gradient, deactivation of thiol-modulated CF_0CF_1 takes several minutes. For this reason only chloroplasts with thiol-modulated CF_0CF_1 are capable of hydrolyzing added ATP after transition from light to dark (22, 23).

Deactivation of the modulated enzyme may proceed with or without reoxidation of the dithiol group (16). Most likely the natural actual oxidant is the oxidized form of thioredoxin. Hence at least four CF_0CF_1 forms may be discerned: the oxidized inactive (E_i^{ox}) and active (E_a^{ox}) enzyme, and the reduced inactive (E_i^{red}) and active (E_a^{red}) enzyme. Due to the lower deactivation rate, the equilibrium of active to inactive enzyme is shifted toward lower proton gradients when CF_0CF_1 is in the reduced form (24). Fast deactivation of the modulated enzyme at light-dark transition is achieved by micromolar concentrations of ADP (25, 26) accompanied with tight binding of the nucleotide molecule to one of the three catalytic sites (27).

In the present paper the interaction of CF_0CF_1 with thioredoxin is analyzed kinetically, and the thioredoxin specificity for thiol modulation is investigated. The efficiencies can be expressed by rate constants for the binding of the different thioredoxins.

EXPERIMENTAL PROCEDURES

Chloroplast thylakoids were isolated from spinach leaves as described in Ref. 28. The reaction medium contained 25 mM Tricine buffer, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, 2 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 50 nM valinomycin (to cancel the electrical potential difference); the total volume was 2.5

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[1] The abbreviations used are: CF_0CF_1, chloroplast ATP synthase complex; CF_1, chloroplast coupling factor 1; ΔpH, transmembrane pH difference; $\Delta\mu H^+$, transmembrane electrochemical proton potential difference; Tr-f, chloroplast thioredoxin-f; Tr-m, chloroplast thioredoxin-m; Trx, *E. coli* thioredoxin; Tricine, *N*-tris(hydroxymethyl)methylglycine.

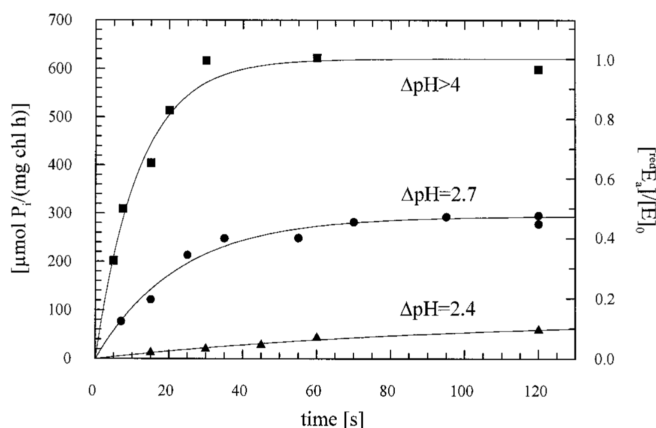


FIG. 1. **Time course of thiol modulation with 3 μM reduced Trx at different extents of the proton gradient.** Thylakoids were preilluminated for 30 s to yield the indicated proton gradients. At zero time 3 μM Trx (reduced by 0.1 mM dithiothreitol) was added while illumination was continued. At the indicated times the ATP-hydrolyzing activity was determined as described under "Experimental Procedures." The term $[E_a^{red}]/[E]_0$ (*right scale*) indicates the relative amount of reduced active enzyme. Further explanations are given in the text. *chl*, chlorophyll.

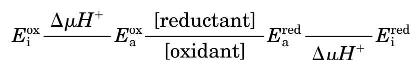
ml. Further additions (dithiothreitol and thioredoxin) are indicated in the legends. The concentration of thylakoids was equivalent to 25 μg of chlorophyll/ml.

Recombinant spinach chloroplast thioredoxins were produced as described in Ref. 29. All experiments were conducted at 20 °C with clamped ΔpH (30), which was measured by the 9-aminoacridine fluorescence quench (31) and monitored as described in Ref. 32. The concentration of 9-aminoacridine was 5 μM. The calibration of the fluorescence signal was done as in Refs. 33 and 34. The activity of reduced CF_0CF_1 was measured by the initial ATP-hydrolyzing activity at ΔpH 0 following illumination (35). Since the portion of oxidized active enzyme is immediately discharged at the time of transition to ΔpH 0 but the reduced enzyme retains activity, the resulting ATP-hydrolyzing activity is a measure of the reduced active form only.

Usually the thylakoids were preilluminated for 30 s to achieve a steady ΔpH. Then thioredoxin + 0.1 mM dithiothreitol was added while illumination was continued. It was ascertained that 0.1 mM dithiothreitol was sufficient to reduce the thioredoxins completely at all concentrations employed. The insignificant ATPase activity induced by this low concentration of dithiothreitol was always subtracted. After different times of illumination, the light was switched off and a mix consisting of 1 mM [γ-^{32}P]ATP and 0.5 μM nigericin (final concentrations) was injected simultaneously. The initial rate of ATP-hydrolyzing activity was ascertained from the contents of released [^{32}P]P_i in samples taken after 3, 6, 9, and 12 s that were deproteinized by 0.5 M $HClO_4$ and analyzed as described in Ref. 36. Rates have been corrected for isotope dilution (less than 5%) that was caused by the ATP-regenerating pyruvate kinase system.

RESULTS

The combined reversible activation/thiol modulation process may be described by Scheme 1.



$$E_i^{ox} \xrightarrow{\Delta\mu H^+} E_a^{ox} \xrightarrow[\text{[oxidant]}]{\text{[reductant]}} E_a^{red} \xrightarrow[\Delta\mu H^+]{} E_i^{red}$$

SCHEME 1

The transition of the active oxidized form to the active reduced form depends on the nature and concentration of the reductant and includes reversible binding of the reductant followed by reduction of the disulfide bridge in the γ subunit. The backward reaction (reoxidation by the oxidized form of thioredoxin (16)) is largely excluded under the employed conditions since in all our experiments the thioredoxins were kept reduced by excess dithiothreitol.

The kinetic measurements of thiol modulation are based on the suppositions that follow. 1) The ATP-hydrolyzing activity at ΔpH 0 reflects the amount of active and reduced CF_0CF_1 since only an active enzyme is able to catalyze ATP hydrolysis and only the reduced enzyme retains its activity after transition to ΔpH 0. 2) Thiol modulation requires the enzyme to be in its activated oxidized state.

TABLE I
Parameters of thiol modulation at different extents of the proton gradient

ΔpH	Final activity of ATP hydrolysis[a]	Relative final activity	Initial increase of ATPase activity	Relative increase of activity
	μmol P_i/(mg chl h)		*nmol P_i/(mg chl s^2 μM Trx)*	
>4	620	1	4.8	1
2.7	294	0.47	1.2	0.25
2.4	84	0.14	0.08	0.017

[a] chl, chlorophyll.

The reduction step is practically irreversible under the employed conditions; all enzyme molecules should become reduced at infinite reaction time independent of the extent of the proton gradient and the thioredoxin concentration. On the other hand, the enzyme activity should depend on the ΔpH to indicate the equilibrium between the reduced active and the reduced inactive form. For a general kinetic analysis we used commercially available Trx from *E. coli*. In Fig. 1, time courses of thiol modulation are shown at 3 μM Trx and three different extents of membrane energization. The final enzyme activities reflecting the equilibrium $E_a^{red} \leftrightarrow E_i^{red}$ increase with increasing light intensity. At ΔpH 2.7 about 50% of the maximal activity is obtained. By acid-base transition Junesch and Gräber (24) found half-maximal activity of the reduced enzyme at ΔpH 2.2. At ΔpH 2.7 they found about 50% of the maximal phosphorylation rate and concluded that in case the enzyme is in the thiol-modulated form, phosphorylation is limited by the catalytic reaction whereas the activity of the ATP synthase is the limiting factor for phosphorylation when the enzyme is oxidized. More recent results, however, have shown that the "activating protons" cannot be discerned kinetically and that the activating protons show the same cooperativity as the "catalytic protons" (37). Hence activation may be a step of the catalytic cycle, and the two processes should have the same ΔpH profile. The apparent difference of the profiles may be due to the fact that the two processes, which have been measured under rather different experimental conditions, are affected differently by factors like ADP or phosphate concentrations (33).

The initial rates of thiol modulation likewise depend on the employed ΔpH (Fig. 1). According to the reaction scheme the rate should be dependent on the concentration of active oxidized enzyme molecules present at the beginning of thiol modulation, *i.e.* on the equilibrium $E_i^{ox} \leftrightarrow E_a^{ox}$ established by preillumination. Accordingly, while the steady-state activities in Fig. 1 represent the activation equilibrium of the reduced form of CF_0CF_1, the initial rates of thiol modulation represent the activation equilibrium of the oxidized form. Compared with the reduced CF_0CF_1 the activation profile of the oxidized enzyme is shifted toward higher ΔpH values (24). The initial rates and the final levels of thiol modulation likewise depend differently on ΔpH in the expected manner (Table I).

According to the results of Junesch and Gräber (24) both activation equilibria should be completely on the side of the active forms at ΔpH ≥ 4. To create clear-cut conditions for the action of thioredoxin, we employed illumination at saturating light intensity to achieve a saturating proton gradient. Saturation was ascertained by the fact that the activity of the oxidized enzyme could not be increased by a further increase of ΔpH. At light saturation the extent of the gradient is well above 4 ΔpH units, but in this range where the 9-aminoacridine calibration curve shows a progressive deflection from linearity, the ΔpH could not be precisely determined.

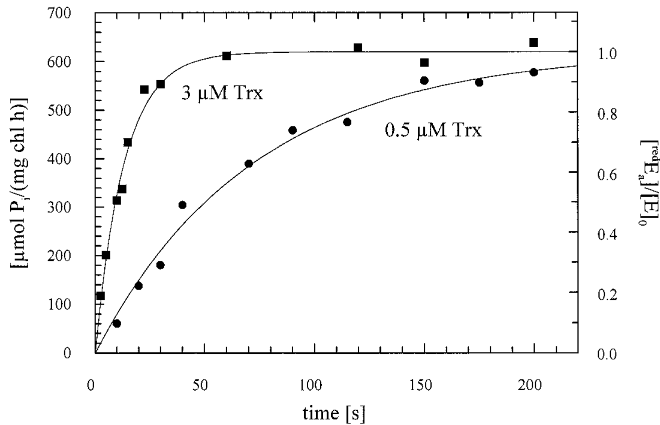


FIG. 2. **Time course of thiol modulation at ΔpH > 4 with 0.5 and 3 μM reduced Trx.** Experimental conditions are as shown Fig. 1.

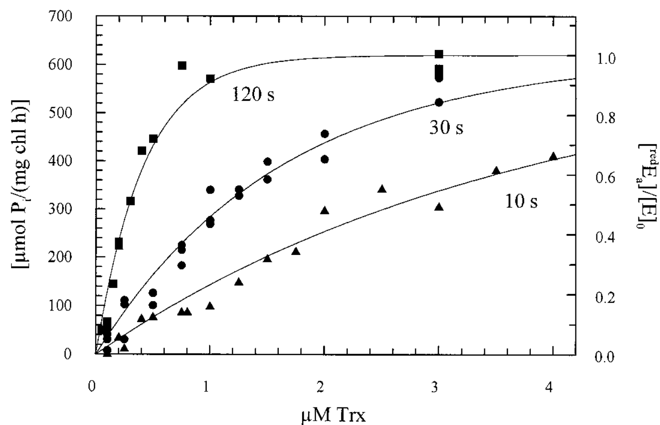


FIG. 3. **Thiol modulation as a function of reduced Trx concentrations at three different reaction times.** Thiol modulation was conducted at ΔpH > 4. Further experimental conditions are as shown in Fig. 1.

Fig. 2 shows at light saturation time courses of thiol modulation with 0.5 and 3 μM Trx. As expected, the curves show different initial slopes depending on the Trx concentration. Whereas the initial rates are proportional to the concentration of thioredoxin both curves level off at the same final activity. This result permits the conclusions that at saturating ΔpH 1) thiol modulation (at least up to 3 μM Trx) is limited by the velocity of Trx binding, 2) the reduction of CF_0CF_1 is practically unidirectional, and 3) the equilibrium $E_a^{red} \leftrightarrow E_i^{red}$ is far on the left side. This context may be described by Scheme 2.

SCHEME 2

The concentration of E_a^{red} at any reaction time t may be expressed by the equation

$$[E_a^{red}] = [E_a^{ox}]_0 \cdot (1 - e^{-k \cdot [\mathrm{Trx}] \cdot t}) \qquad \text{(Eq. 1)}$$

where $[E_a^{ox}]_0$ means the concentration of E_a^{ox} at the reaction start. As pointed out above, $[E_a^{ox}]_0$ under the employed conditions is assumed to be equal to the total CF_0CF_1 population. Using the correct molecular weight of the ATP synthase, the amount of CF_0CF_1 in the thylakoid membrane is 1 nmol/mg of chlorophyll (38). 1 nmol of CF_0CF_1 per mg of chlorophyll therefore can be expressed by the maximal ATPase activity obtained at infinite thiol modulation time. The curves in Fig. 2 present the best fits according to the above equation. The obtained second order rate constants are k = 0.030 s^{-1} μM^{-1} (at 0.5 μM Trx) and 0.028 s^{-1} μM^{-1} (at 3 μM Trx), respectively.

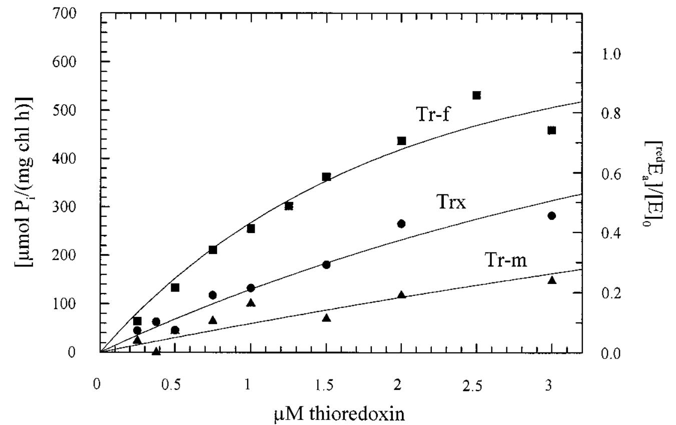


FIG. 4. **Thiol modulation as a function of the concentrations of reduced Tr-f, Trx, and Tr-m.** Thiol modulation was conducted at ΔpH > 4. The ATP-hydrolyzing activity was determined after 10 s of thiol modulation. Further experimental conditions are as shown in Fig. 1.

TABLE II
Second order rate constants for thioredoxins and dithiothreitol in thiol modulation

Reductant	k
	s^{-1} μM^{-1}
Tr-f	0.057
Trx	0.024
Tr-m	0.010
Dithiothreitol	$1.02 \cdot 10^{-6}$

Equation 1 predicts that the degree of thiol modulation is an exponential function of both the reaction time and the thioredoxin concentration. In an experiment shown in Fig. 3 the degree of thiol modulation as measured by the ATPase activity is plotted as a function of the thioredoxin concentration at three different reaction times. The curves were again fitted according to Equation 1 and yielded the rate constants 0.026 s^{-1} μM^{-1} (at 10 s), 0.021 s^{-1} μM^{-1} (at 30 s), and 0.020 s^{-1} μM^{-1} (at 120 s reaction time), respectively. The mean value including standard deviation of k for Trx from all experiments was 0.024 ± 0.003 s^{-1} μM^{-1}.

Futhermore the efficiencies of the chloroplast thioredoxins Tr-m and Tr-f in thiol modulation are compared with Trx and dithiothreitol. Concentration dependences at 2 min modulation time indicated that under all conditions the same final activity is attained (not shown). In Fig. 4 ATPase activities are shown after a reaction time of 10 s in the presence of different concentrations of Tr-f, Trx, and Tr-m. The rate constants calculated from the best fits are summarized in Table II. The results indicate that among the three thioredoxins Tr-f is most efficient followed by Trx and Tr-m. The rate constant for dithiothreitol is at least 4 orders of magnitude lower (Table II).

DISCUSSION

Thiol modulation of CF_0CF_1 *in membrana* requires illumination of the thylakoids and a dithiol reductant to reduce the disulfide bond in subunit γ. Originally this complex interplay was expressed by the term "light-triggered ATPase" (22, 39). Now it is clear that light is necessary for CF_0CF_1 activation and that it is the transmembrane proton gradient that is essential (1). As a consequence of energization a series of conformational changes occur in CF_0CF_1. Changes in the region of the nucleotide binding sites located in the α-β subunits lead to the release of tightly bound adenine nucleotides (1, 2, 28), ϵ subunit is available for an antibody (40), and Lys-109 of the ϵ subunit becomes accessible to chemical modification by pyridoxal 5′-phosphate (41). Likewise the so-called "light site" of the γ

subunit, a cysteine residue in position 89, becomes accessible to modification by *o*-phenylenedimaleimide (42). Similarly the target sequence for thiol modulation, the disulfide bridge formed between Cys-199 and Cys-205 in the γ subunit, is exposed. This domain, which is hidden in the inactive enzyme, becomes available to tryptic cleavage upon activation (43) and accessible to reduction by dithiol reductants. At full CF_0CF_1 activation the pure kinetics of reduction can be measured, and a second order rate constant can be determined. The rate constant depends of course on the nature of the reductant and is much higher for the thioredoxins than for dithiothreitol.

In the basic work of Mills *et al.* (20) it was shown that Tr-f is active in unmasking the ATPase in thylakoids in the light. The result reported here suggests that Tr-f (rather than Tr-m) is indeed the natural reductant of CF_0CF_1 in the chloroplast. Tr-m is even less effective than Trx from *E. coli*. To our knowledge this is the first clear comparative kinetic study of thiol modulation of CF_0CF_1 *in membrana* with the natural chloroplast thioredoxins. Galmiche *et al.* (19) compared the effects of Tr-m, Trx, and human thioredoxin on the activation of isolated CF_1 and found no differences between the former two, but the human thioredoxin was 10 times less effective.

In fact the differences in efficiency between Tr-f, Trx, and Tr-m are apparent at short reaction times. This is due to the fact that reduction of γ is virtually an irreversible reaction provided that the thioredoxins are kept reduced by excess dithiothreitol. Theoretically the process includes two successive reactions, reversible binding of thioredoxin to CF_0CF_1 followed by the reduction step itself. The formation of an intermolecular complex between isolated CF_1 and Trx was measured by Dann and McCarty (16) by using fluorescent probes. They found dissociation constants in the range of 1 μM. Nevertheless the total process including reduction can be described by a single irreversible second order process suggesting that the irreversible reduction step is fast compared with thioredoxin association/dissociation. Accordingly the efficiency may be expressed by the second order rate constant for the association of thioredoxin with CF_1. Mathematical simulations of models assuming the reduction step as the rate-limiting reaction could not well explain our experimental data.

Compared with dithiothreitol all thioredoxins are by a factor of at least 10^4 more efficient suggesting that the specific structure or orientation of the dithiol group in the thioredoxins bound to CF_0CF_1 is essential for high reactivity. The sequences of Tr-f and Tr-m on one hand and Tr-f and Trx on the other hand contain about 30% identical amino acids, but the highest homology is found between Tr-m and Trx (46% identical positions). Accordingly the reactivities in thiol modulation are not well substantiated by the overall primary structures. As the three thioredoxins have the same sequence motif in the region of the reactive dithiol group (WCGPCK), the reactivity might be based on specific amino acid domains located on the protein surface. The three-dimensional structure of Trx from *E. coli* shows a central twisted β sheet composed of five β strands surrounded by four α helices. The domain containing the reactive cysteines Cys-32 and Cys-35 is in helix α_2. Eklund *et al.* (44) proposed that the hydrophobic flat area around the reactive cysteines may form contact with the target protein. The differences in efficiency may be referred to as slight differences in the conformation of this contact area. Recent crystallographic analyses of the chloroplast thioredoxins confirm that they have the same general architectures as the other analyzed thioredoxins.[2] However, due to their sequences the two chloroplast proteins show quite different surface structures. The surface area of Tr-f around the accessible active site cysteine is not as flat and hydrophobic as that of Trx but quite structured and surrounded by positive and negative charges. These charges are probably instrumental for the proper orientation during the protein-protein interaction. The corresponding surface area of Tr-m resembles much more the one of Trx, except for one boundary area. The fact that Tr-m is less efficient in thiol modulation than Trx may be due to some topological difference between these on the whole rather similar proteins. It might also be due to the replacement in the boundary area of Tr-m of three hydrophobic residues present in Tr-f and Trx by three charged residues: V86(R)/A87(K)/A88(E) (notation of *E. coli*-Trx; the corresponding amino acids of Tr-m are in parentheses). These charges may reduce the binding of Tr-m to the coupling factor and therefore be responsible for its lower efficiency compared with Tr-f and Trx.

[2] G. Capitani, Z. Markovich-Hously, G. del Val, P. Schürmann, and J. N. Jansonius, manuscript in preparation.

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