On the Possibility of High-Precision Photothermal Microeffects and the Measurement of Fast Thermal Denaturation of Proteins

Gereon Hüttmann and Reginald Birngruber

Abstract—**The precision of laser-induced effects is often limited by thermal and thermomechanical collateral damage. Adjusting the pulsewidth of the laser to the size of the absorbing structure can at least avoid thermal side effects and facilitates a selective treatment of vessels or pigmented cells. Further extending the precision of thermal effects below cellular dimensions by using nanometer sized particles could open up new fields of applications for lasers in medicine and biology. Calculations show that under irradiation with nano- or picosecond laser pulses gold particles of submicrometer size can easily be heated by several hundred K. High temperatures have to be used for subcellular thermal effects, because heat confinement to such small structures requires the thermal damage to occur in extremely short times. Estimating the denaturation temperature by extrapolating the Arrhenius equation from a time range of minutes and seconds into a time range of nano- and picoseconds leads to temperatures between 370 K–470 K. There is evidence that in aqueous media, due to the surface tension, these temperatures can be generated at the surface of nanometer sized particles without vaporization of the surrounding water.**

In order to show whether or not an extrapolation of the damage rates over six to nine orders of magnitude gives correct data, a temperature-jump experiment was designed and tested which allows to measure denaturation rates of proteins in the millisecond time range. Denaturation of chymotrypsin was observed within 300 μs at temperatures below 380 K. The rate constants for the unfolding of chymotrypsin followed the Arrhenius equation up to rates of 3000 s^{-1}.

Index Terms—**Laser medicine, microeffects, protein denaturation, thermal effects.**

I. Introduction

LASERS are used in a multitude of therapeutic applications because of the high precision, which can be achieved in destroying or altering tissue. This precision is normally limited by the size of the laser focus and by collateral thermal or mechanical damage. Tissue structures can be targeted either directly by a tightly focused beam or by selectively marking the target structure with absorbing dyes or particles which extract the energy from a broad illuminating beam. With the first approach, the precision is ultimately limited by light diffraction. A focus size not smaller than half a micrometer can be realized with visible light in a microscopic setup [1], [2]. For medical applications *in vivo* this precision is often not possible. The second approach facilitates, in principle, a much higher precision, which is only limited by the size of the absorber and by collateral damage which may occur upon irradiation. Moreover, simultaneously treating a large number of target sites is possible by irradiating with a broad irradiation beam.

By adjusting the laser pulsewidth to the thermal relaxation of the target, the spatial extension of thermal damage can be controlled. This was used for selective thermolysis of vessels [3]–[5] and selective coagulation or disruption of pigmented cells [6], [7]. Laser treatments of ocular diseases, which rely on a selective destruction of melanin containing cells, are currently being tested in clinical studies [8], [9]. To create photothermal microeffects within subcellular or macromolecular structures would open up new fields of applications for lasers in medicine and biology. Even selective targeting of single proteins could be possible with this approach.

The first part of this paper discusses the feasibility of photothermal effects with subcellular spatial extension. Since the denaturation kinetics at high temperatures is important for extremely localized thermal effects, the second part of the paper presents a temperature-jump experiment, which measures denaturation rates with a high time resolution. Results of these measurements with the protein chymotrypsin are reported.

II. Feasibility of High Precision Photothermal Microeffects

Prerequisite for localized thermal effects is the use of short heating times. Energy should be applied in times shorter than the thermal relaxation time τ of the target structure [3], which depends on its diameter d and thermal diffusivity α:

$$\tau = \frac{d^2}{27\alpha}. \tag{1}$$

In tissue or water microsecond laser pulses are necessary to confine the thermal energy to 1 μm (Fig. 1). Further decreasing the damage range by two orders of magnitude to 10 nm requires laser pulses in the picosecond range. Therefore, the thermal effect at the target has to occur on this extremely short time scale. The feasibility of high precision thermal effects is related to two questions.

1) Is it possible to achieve thermal denaturation of biomolecules within nano- or picoseconds?

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The authors are with the Medical Laser Center Lübeck, D-23562 Lübeck, Germany.

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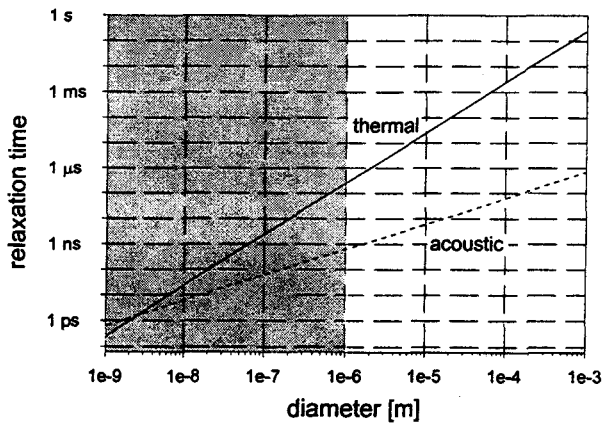

Fig. 1. Thermal and acoustic relaxation time of a spherical volumes of water or tissue with diameters ranging from 1 nm to 1 mm. The shaded area shows the range, which is important for subcellular microeffects.

2) Is it possible to generate locally sufficiently high temperatures by nanometer absorbing structures?

The first question is about the kinetics of thermal damage. In principle, thermal effects can be caused by two different mechanisms: phase changes at a certain threshold temperature or a kinetic mechanism with damage depending on time and temperature. Thermal damage of proteins, cells or tissues was shown to be a process with a strong logarithmic dependence of the damage rate k on the temperature T, which can be described by the Arrhenius equation [10]–[12]

$$k(T) = A_0 \exp\left(-\frac{E_a}{RT}\right). \tag{2}$$

A_0 and E_a are the two characteristic parameters for the denaturation process which are usually interpreted as a frequency factor and an activation energy. R is the universal gas constant. The extent of thermal damage is given by the damage integral Ω which can be calculated by an integration of $k(T)$ over the time-temperature history:

$$\Omega = \int k(T)\, dt. \tag{3}$$

Proteins are the main targets for microeffects because they play a key role in cellular processes and are responsible for thermal damage in cells [13]–[15]. Extremely high rate constants k are necessary to create thermal damage in nano- or even picoseconds. So far, no information is available whether these damage rates can be achieved and which temperatures would be needed, because data about thermal effects on proteins, cells and tissues were obtained in the range of 10^3–10^{-1} s [10], [14], [16]–[20]. In this time range, experiments showed a strong temperature dependence of the rate constants in accordance with the Arrhenius law. An extrapolation of these data to higher temperatures suggests that a thermal denaturation in 100 ps (damage rates of 10^{10} s^{-1}) should be possible at temperatures between 370 K–470 K (Fig. 2). The bold part of the Arrhenius lines indicates the range in which the Arrhenius parameters were measured.

These temperatures are well above the boiling point of water at ambient pressure. Experiments have shown that an evaporation at the surface of micro- and nanoparticles can cause rapidly expanding vapor bubbles [21] and significant mechanical damage [22].

Although this thermomechanical effect can be fairly localized—selective damage of single cells has been demonstrated [7], [22]—the collateral damage is more extensive than it could be with a purely thermal mechanism.

Studying laser induced bubble formation at melanosomes [23], a significant increase in the threshold temperature for evaporation at the particle surface was observed. An explanation for this effect is the contribution of the surface tension S of water to the ambient pressure when a spherical bubble of a diameter d is formed

$$p_s = 4\frac{S}{d}. \tag{4}$$

This additional pressure p_s raises the temperature at which water evaporates. A significant increase in the threshold temperature for bubble formation is therefore expected for small particles, since bubbles have to start with a size equal or smaller than particle size. In order to compare the temperatures needed for thermal effects and bubble formation, the boiling temperature of water (T_{boiling}) at a pressure of $p_s + 1$ bar was plotted in Fig. 2 against the particle diameter (right ordinate). Diameter and denaturation rates can be related via the thermal relaxation time of the particle. For high precision microthermal effects, laser pulsewidth, and heating time should not be larger than the thermal relaxation time τ of the particle. If thermal properties of water are assumed, τ can be easily calculated by (4). Irradiating the particles, the temperature must be high enough to create damage within τ. Therefore, the rate constants for damage have to be at least $1/\tau$. In order to compare the temperatures for denaturation and bubble formation, the axis of the particle diameter was scaled in such a way that the reciprocal of the damage rate constants (left ordinate) is the thermal relaxation time of a particle with the given diameter. Thermal properties of water were assumed for the absorbing structure and the surrounding medium.

In Fig. 2, the extrapolated temperatures necessary for thermal microeffects in the different biological systems can be compared with the expected threshold temperature for bubble formation at different particle diameters.

For thermal effects with a spatial extension of centimeters or millimeters, the tissue can be heated for minutes or seconds and only moderate temperatures well below the boiling point of water are required. For targeting single cells, the damage has to be restricted to 5 to 10 μm, and the temperatures for thermal damage and bubble formation are very close together. Surface tension does not yet play a significant role. The mechanisms of retinal damage after laser expositions with different pulsewidths support this interpretation. Millisecond and submillisecond exposures result in thermal damage [14], whereas with single nano- and microsecond pulses mechanical damage [8], [24] and bubble formation are observed. Thermal damage of RPE cells with microsecond pulses is only possible with repetitive irradiation utilizing the additivity of thermal effects to reduce the necessary temperature [6]. With nanometer particles the surface tension should contribute significantly to

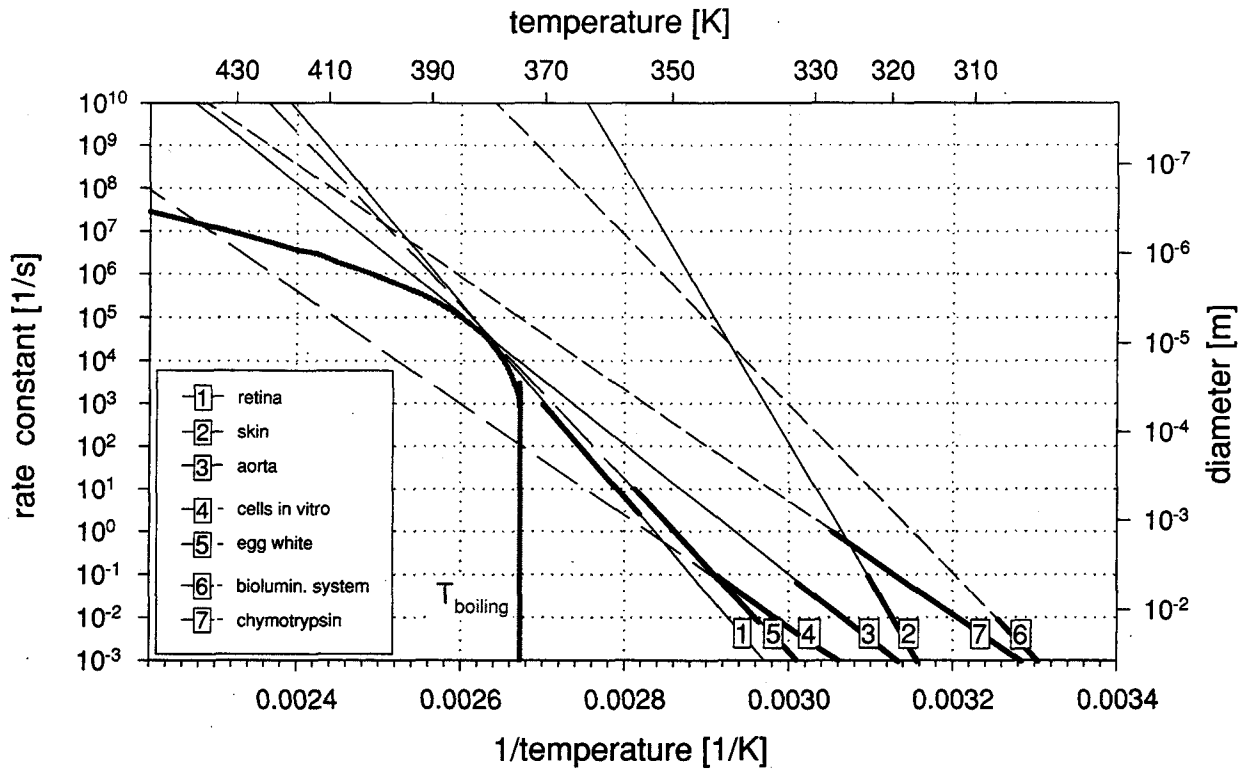

Fig. 2. Arrhenius plot for thermal damage of different tissues (retina [14], skin [16], aorta [17]), cells [18], and proteins [10], [20] (left ordinate). Lines were calculated from the Arrhenius parameters A_0 and E_a derived from measurements in the temperature range, which is indicated by the bold lines. Boiling temperature of water (T_{boiling}) was calculated considering the influence of the surface tension of water on gas bubbles of the given diameter (right ordiante). See text for more information.

the pressure inside the bubble and a drastic increase in the threshold temperature for mechanical damage is expected as the particle size is reduced.

Although it is thought that nano- and picosecond laser pulses are connected with photomechanical effects, the effect of surface tension and the use of pulse series could allow thermal effects with nanometer precision.

The second difficulty concerning thermal microeffects is how to heat submicrometer volumes efficiently by laser irradiation. This requires a high absorption cross section, an efficient conversion of the absorbed energy to heat within the laser pulsewidth and a high photostability of the absorbing structures.

Calculations based on Mie scattering [25] and heat diffusion show that nanometer-sized particles can be heated very efficiently by laser irradiation. This is demonstrated for spherical gold particles at a wavelength of 524 nm. Gold particles are interesting candidates for investigating microeffects since their optical and thermal properties are well characterized and they can be coupled easily to antibodies or proteins. Although gold is reflecting macroscopically, most of the incident light nanometer-sized particles of gold are very efficient absorbers. For particle sizes between 10–200 nm the absorption cross section σ exceeds the geometric cross section A (3.2 times at a diameter of 35 nm). Even at a diameter of 1 μm the absorption cross section is still half the particle cross section. The ratio between A and the particle volume increases as the particle gets smaller. Therefore, the density of absorbed energy, which determines the particle temperature if heat diffusion is neglected, increases if the ratio between σ and A is constant. This is no longer true for a diameter considerably smaller than the wavelength of the irradiating light (<10 nm). Here, the absorption cross section σ is proportional to the particle volume [26] and therefore the density of absorbed energy does not depend on the particle size.

Heat diffusion was incorporated into the temperature calculations by using an analytical formula for the temporal and spatial temperature distribution in the surroundings of a homogeneously heated sphere, which is embedded in an infinite medium of different thermal properties [27]. The surface temperature was calculated for a radiant exposure of 1 mJ/cm^2 and pulsewidths of 1 ps, 1 ns, and 1 μs (Fig. 3). The time-dependent solution was constructed for a rectangular-shaped laser pulse [11], and thermal properties of water were assumed for the surrounding medium.

With picosecond pulses a temperature increase of more than 200 K per mJ/cm^2 is achieved for particle sizes between 10–100 nm. Only below 10 nm, heat diffusion reduces the surface temperature significantly. With nanosecond pulses, heat diffusion already starts to reduce the surface temperature for particles smaller than 200 nm, but high temperatures can still be achieved. Microsecond pulses are not adequate for heating submicrometer particles because at reasonable radiant exposures they are several orders of magnitude less effective than nano- and picosecond pulses. Although the gold particles are not necessarily heated homogeneously when irradiated, the

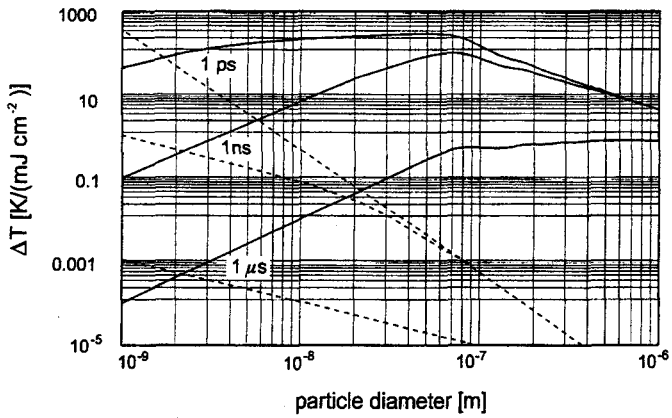

Fig. 3. Calculations of the temperature increase on the surface of gold spheres with different diameters (—). An irradiation with 1 mJ/cm^2 at 524 nm was assumed. For comparison temperature increases caused by irradiation of a single-dye molecule (molar extinction coefficient $10^6 M^{-1}$ cm^{-1}) were calculated in a distance from the molecule, which is half the particle diameter (- - -). Both, particle and dye molecule were embedded in water.

nearly three orders of magnitude higher thermal diffusivity of gold compared to water rapidly equalizes the temperature distribution inside the particle.

Due to their metallic properties, photobleaching and thermalization of the absorbed energy are not expected to limit the temperature increase. There is experimental evidence that gold particles can even be melted in water by irradiation with nanosecond visible light [28]. High irradiances of 1 GW/cm^2 for picosecond pulses and 10 MW/cm^2 for nanosecond pulses are needed for a temperature increase of 200 K. However, these values are well below the threshold of optical breakdown in water, which is 850 GW/cm^2 and 45 GW/cm^2 for 3 ps and 6 ns pulsewidths, respectively [29].

A dye molecule does not have the absorption cross section, the fast thermalization of the absorbed energy and the photostability which are needed for thermal microeffects. Even with a high molar extinction coefficient of 10^6 M^{-1} cm^{-1} the absorption cross section is only 0.4 nm^2, which is comparable to that of a 1.8-nm gold particle. With a solution of the heat diffusion problem for a point heat source [30], the temperature increase at different distances from the molecule was calculated (Fig. 3). At a distance of 1.5 nm from the dye molecule and a pulsewidth of 1 ps, a radiant exposure of 10 mJ/cm^2 is needed for a temperature increase of 100 K and approximately 100 photons have to be absorbed by the molecule during the laser pulse, if visible green light is used.

This requires an excellent photostability and relaxation times of the excited states which are involved in the absorption process in the range of femtoseconds. In general, the lifetime of the first excited state is in the range of nano- or picoseconds. The attempt to pump enough energy into the molecule within 1 ps would, therefore, most likely result in a decrease in the absorption due to transient bleaching and an excitation to higher states which probably leads to photochemical modification of the dye molecule and of adjacent structures. It is therefore expected that the attempt to create photothermal effects with single dye molecules will eventually produce photochemical damage. Photochemical destruction of proteins was observed with malachite green coupled to antibodies under irradiation with nanosecond laser pulses [31], although malachite green is very efficient in converting light to heat.

In conclusion, high temperatures can be generated on the surface of gold particles under irradiation with nano- and picosecond laser pulses with a radiant exposure of tens or a few hundreds mJ/cm^2. The crucial point for the feasibility of thermal microeffects is the denaturation kinetics of proteins. Therefore, an experiment was designed that allows the direct measurement of the protein denaturation kinetics with submillisecond time resolution. The basic idea is to instantaneously heat a macroscopic volume of a protein solution and to track the denaturation of the proteins with the necessary time resolution by optical means [32]. Such temperature-jump experiments were successfully used in kinetic studies using either light or an electric current to heat the sample volume [33], [34]. Although protein dynamics has been studied with nano- and even picosecond resolution [35]–[37], little is known about protein unfolding and denaturation at very high temperatures.

III. Materials and Methods

The setup for the temperature-jump experiment is shown in Fig. 4. The protein solution was heated by infrared pulsed radiation at 2.1 μm from a self-built Holmium laser (CTH:YAG with 300 μs pulsewidth [38]). The infrared radiation is directly absorbed by the water molecules and therefore complications due to photochemistry which may by induced by high irradiance visible light are prevented. The absorption of the 2.1-μm irradiation in water offers a good compromise between the size of the probe volume and the necessary pulse energy [32]. With this simple and reliable setup, temperature jumps of more than 80 K are possible. By means of a beam splitter, the emission of the Holmium laser was coupled into two 600-μm low-OH fibers which delivered the IR pulses to the probe volume. The exit surfaces of both fibers were positioned at a distance of 300 μm parallel to each other.

Denaturation of chymotrypsin was measured by observing the changes of the UV absorption. Light from a deuterium lamp was guided by a 200-μm fiber to the probe volume. This fiber was positioned perpendicular to both 600-μm fibers. After crossing the gap between these fibers, the UV light was gathered by another 500-μm fiber and directed onto two photomultipliers (Hamamatsu) via a semitransparent mirror. All fibers had a NA of 0.2 and were manufactured by Ceram Optec. For each temperature jump, a fresh drop of protein solution was suspended between the four fibers. Absorption was measured at two wavelengths, which were selected by dielectric filters in front of the photomultipliers (280-nm bandpath, 30-nm bandwidth, Omega Optical and 320-nm bandpath, 12-nm bandwidth, Laser Components).

At 280 nm, changes in the protein absorption due to denaturation of chymotrypsin were measured [39], [40]. Transmission changes measured at 320 nm, a wavelength at which chymotrypsin does not absorb, were used to correct for thermally induced changes in the index of refraction. The normalized signal at 280 nm (S_{280}) depends on absorption changes $\Delta\alpha$

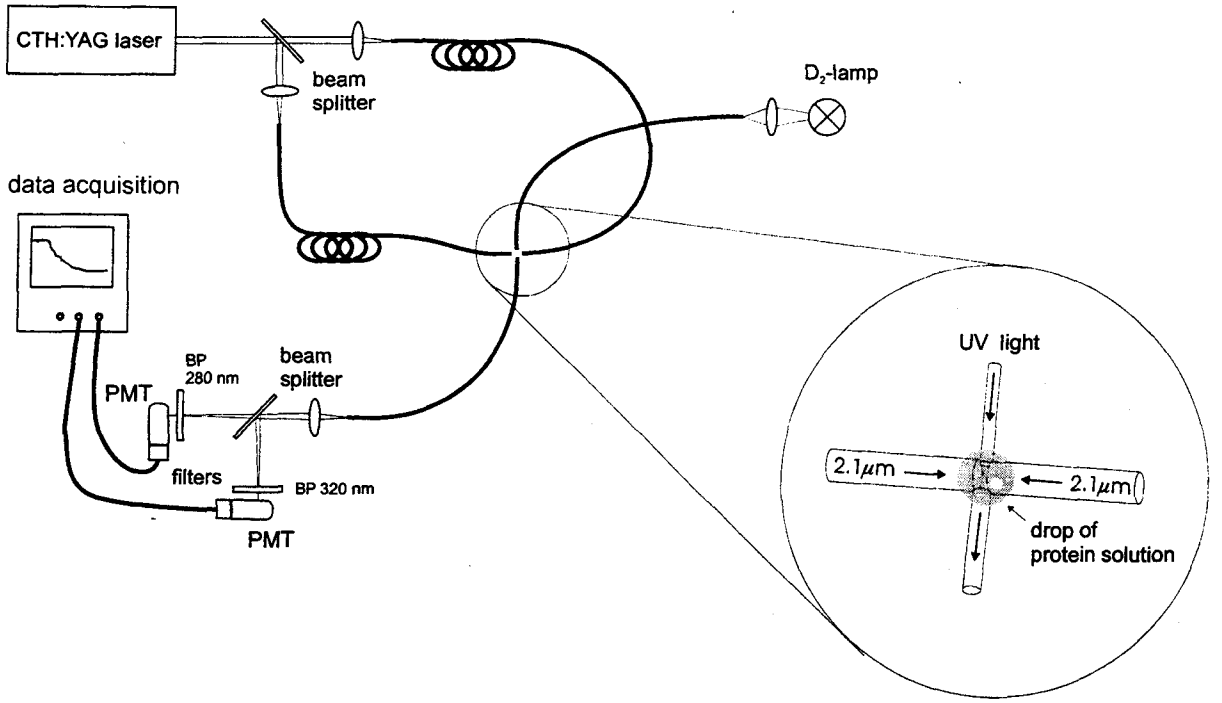

Fig. 4. Setup for the measurement of fast protein denaturation. The Holmium laser heats the small volume, which contains the protein solution via two fibers. Denaturation was detected by changes of the UV-absorption, measured by two photomultipliers, which were equipped with UV bandpass filters.

of the protein solution, the path length d, and the transmission changes $\Delta\tau$ caused by variations in the index of refraction

$$S_{280} = \exp(-\Delta\alpha d - \Delta\tau_{280}). \tag{5}$$

At 320 nm changes of the measured signal are only related to temperature-induced transmission changes:

$$S_{320} = \exp(-\Delta\tau_{320}). \tag{6}$$

$\Delta\tau$ was similar at both wavelengths and all signal changes were small enough (<7%) to justify a linear approximation of the exponential function. Hence, the difference of the normalized signals was only given by the absorption change $\Delta\alpha$ of the protein solution multiplied by the length d of the probe volume

$$S_{320} - S_{280} = \Delta\alpha d. \tag{7}$$

Experiments with distilled water proved that by subtracting S_{280} from S_{320} signal changes not related to protein absorption can be canceled nearly completely.

The fastest denaturation rate which could be measured was limited by the slope of the temperature jump given by the pulsewidth of the Holmium laser (300 μs). The lowest rate was limited by the cooling of the probe volume, which lasted approximately 100 ms. Allowing for a temperature decrease of only 5%, the time in which the denaturation of the proteins can be followed, is limited to 5 ms. Therefore denaturation rates between 100–3000 s^{-1} can be measured with reasonable accuracy.

Chymotrypsin (Sigma) was used without further purification. Because of the short absorption pathway a rather concentrated solution of 5 mg/ml was used. The pH was adjusted to 2.5 by adding an appropriate amount of HCl.

The rate constants of the denaturation were determined by fitting a sum of an exponential function and a constant to the measured absorption changes:

$$S = S_0(1 - \exp(-kt)) + c. \tag{8}$$

The parameters S_0, k, and c were adjusted by a nonlinear fit algorithm in order to minimize the squared difference between the measured data and the convolution of (8) with the shape of the IR laser pulse.

The temperatures were calculated from the IR energy which was delivered to the probe volume and the absorption coefficients of water which was measured to be 2.5 mm^{-1}. The energy of the IR pulses was measured by a pyroelectric energy meter (Gentec). The temperature dependence of the water absorption at 2.1 μm, which is less than 10% in the range between 283 K–353 K [41], was neglected when calculating the jump temperature. The accuracy of the calculated temperatures and the homogeneity of the heating of the probe volume were checked by the onset of vapor formation in the probe volume when heated by the laser pulses above the boiling point of water.

IV. Results and Discussion

Denaturation kinetics of chymotrypsin was measured for different temperatures between 330 K–370 K. After heating the protein solution by the infrared laser pulse, a change in protein absorption was observed that consisted of two phases (Fig. 5). A rapid change within the time resolution of the setup was followed by a further exponential decrease of the

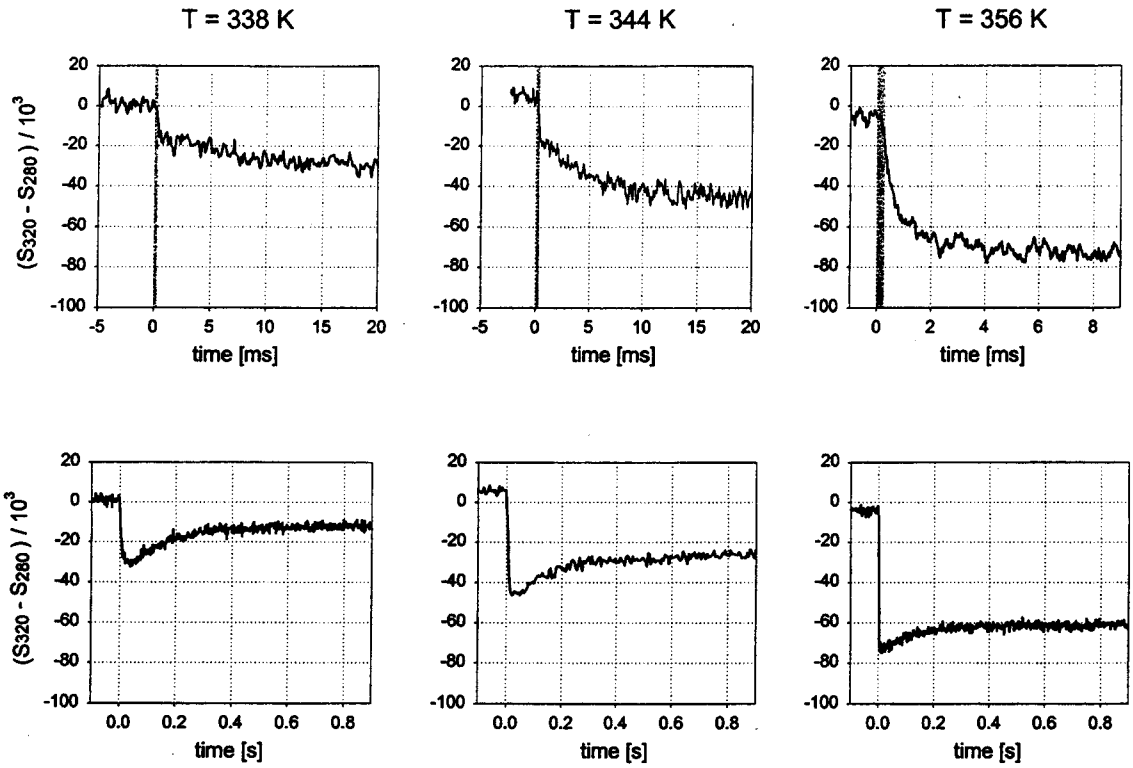

Fig. 5. Measured absorption changes of chymotrypsin ($S_{320} - S_{280}$) for three different jump temperatures. The signals were measured on a fast (upper row) and a slow (lower row) time scale. The length of the laser pulse is indicated by the bar at time zero. Note the different time scales for different jump temperatures.

measured signal. As the jump temperature was increased, the exponential absorption change occurred faster and had a larger amplitude. Measurements on a longer time scale revealed that, to a certain degree, the absorption changes were reversible. As the probe volume cooled down, the transmission increased again, but did not return to its starting value. In Fig. 5, at a jump temperature of 338 K, the absorption returned to 50% of the temperature induced signal change, whereas at 356 K only 20% of the absorption changes were reversible.

Denaturation of chymotrypsin is accompanied by a decrease of UV absorption between 230–300 nm [39]. At low protein concentrations this process is reversible and the protein can fold back to its native structure with high efficiency. At the high concentrations which were used in the temperature-jump experiments the unfolded proteins aggregate and the denaturation process becomes partly irreversible.

In experiments with chymotrypsinogen A which can be converted to chymotrypsin by a bond cleavage, two exponential components were observed in the denaturation kinetics [42]. They were attributed to the unfolding of the native protein N and to the isomerization of a proline residue which gives further unfolded species U', with spectroscopic properties similar to U [20], [43]. Even a simple model with only two unfolded species U and U' and an irreversible aggregation of the unfolded proteins results in a quite complicated kinetic model. The unfolded proteins may aggregate with U, U' or with the native form N

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} U \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} U' + \begin{matrix} N \\ U \\ U' \end{matrix} \overset{k_{3i}}{\rightarrow} \begin{matrix} U'U' \\ U'U \\ U'N' \end{matrix} . \tag{9}$$

A complete analysis of the denaturation kinetics is quite complex and beyond the scope of this paper. However, at the high jump temperatures which were used in these experiments the equilibrium between native and unfolded protein is far on the unfolded side, which implies that k_{12} is much larger than k_{21}. Therefore the back reaction from U to N can be neglected. In this case the apparent rate constant k, which is calculated directly from measured absorption changes, is similar to k_{12}, the rate constant for the primary unfolding step. The unfolding rate k_{12} ultimately limits the time in which a denaturation can occur. Back reaction, isomerization, and aggregation mainly influence the yield of denatured proteins.

The origin of the fast absorption change during the laser pulse is not clear. It could be the result of a fast additional step in the unfolding process or of temperature-induced absorption changes of chymotrypsin. Fitting an exponential function according to (8) to the measured absorption changes $S_{320} - S_{280}$ gave the apparent rate constant k. In the range from 100 to 3000 s^{-1}, in which reaction rates can be measured quite accurately, the Arrhenius plot of the rate constants gives a straight line (Fig. 6). The measured rate constants fit quite well to measurements of chymotrypsin denaturation in the time range of seconds and minutes [39], which are shown as solid lines for different pH values in Fig. 6. Neglecting U', k is determined by the sum of the rates for unfolding (k_{12}) and refolding (k_{21}). At lower temperatures, k is therefore dominated by k_{21}, whereas at high temperatures the unfolding rate k_{12} dominates the kinetics of the absorption changes. From the laser temperature jump experiment and the low temperature

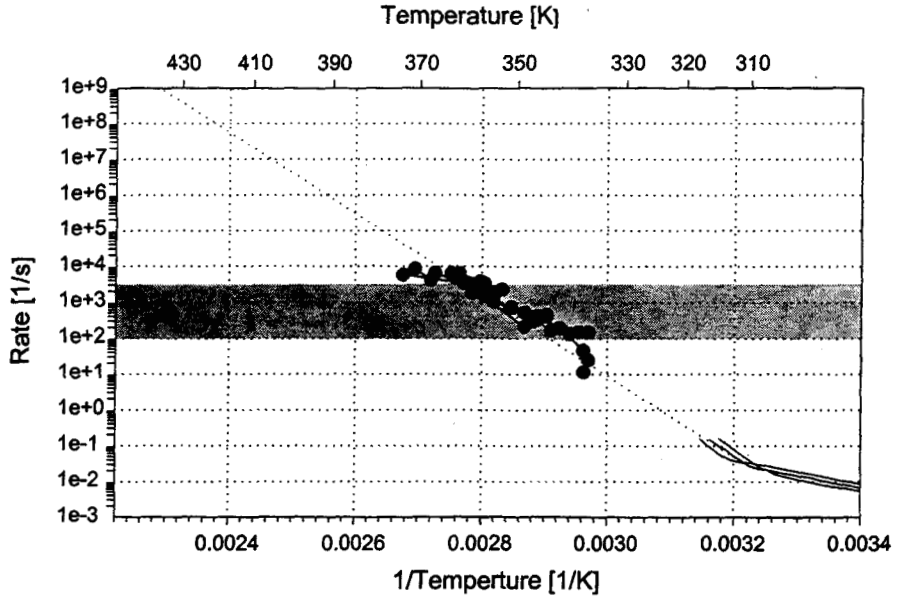

Fig. 6. Arrhenius plot of the rate constants (•) measured by the temperature-jump experiment of chymotrypsin. The shaded area is the range in which the denaturation rates can be measured with reasonable accuracy. For comparison, unfolding rates measured at lower temperatures [39] for three different pH values (2.11, 2.33, and 2.51) are shown (—).

data, it can be concluded that for the first unfolding step of chymotrypsin, the Arrhenius equation is valid at least up to a time range of a few hundred microseconds.

V. Conclusion

The attempt to use the principle of selective thermolysis in order to push the precision of laser induced tissue effects into the macromolecular range, raises a number of interesting questions regarding the denaturation kinetics of biomolecules, the energy deposition in chromophores, pigments, and other absorbers and the evaporation of water in nanometer sized volumes. Thermal effects, which are localized on a nanometer scale, require a heating of the target for only pico- or nanoseconds. Hence, high temperatures have to be created in order to speed up the rates of the thermal damage process. The absorber, which is needed to transfer the energy from the electromagnetic field to the target, must have a large absorption cross section, subpicosecond thermalization of the absorbed energy, and a very good photo- and thermal stability. It is expected that only solid-state absorbing particles (e.g., metal spheres, melanin, graphite, or iron oxide particles) can be used as such an energy acceptor for thermal microeffects. Dye molecules do probably not have the required photostability and will, therefore, rather produce photochemical damage than photothermal effects. Calculations have shown that gold particles are very efficient absorbers, which can easily be heated by several hundred kelvin using nano- and picosecond pulses of visible green light.

The general observation that by decreasing the heating time of tissue structures from milliseconds to micro- and nanoseconds, the damage mechanism changes from thermal denaturation to mechanical damage caused by explosive evaporation, spallation, or shock waves, seems to speak against thermal microeffects on a macromolecular scale. However, two effects may help. First, the additivity of thermal effects allows to apply multiple pulses repetitively and thereby to reduce the necessary temperature without compromising the spatial confinement of the thermal effects. Second, the surface tension of water increases the threshold for evaporation considerably as the particle size falls under 1 μm. This may open a new window for localized thermal effects in the nanometer range. Down to a particle size of a few nanometers, the acoustic relaxation time is shorter than the thermal relaxation time, and therefore damage by acoustic waves can be neglected.

One key issue for the feasibility of thermal microeffects is the denaturation kinetics of proteins at high temperatures. A comparably simple temperature-jump experiment allows to follow directly the protein denaturation with a time resolution of several hundred microseconds. Mechanisms of protein unfolding and denaturation can be studied with this setup and the validity of the Arrhenius law can be tested. It was shown that the unfolding of chymotrypsin follows the Arrhenius equation at least up to rates of 3000 s^{-1}. The denaturation kinetics of more complicated systems like egg white can be measured as well and, in principle, also tissue denaturation could by measured, if it is connected to changes in optical tissue properties. An improvement of the time resolution up to 1 μs seems to be possible by incorporating a Q-switch into the infrared laser. This is the interesting time range for the denaturation of proteins in cells and tissues, which are heated by single melanosomes or by irradiating a tissue surface with strongly absorbed pulsed irradiation (e.g., 193 nm or 2.9 μm).

Photochemical, photothermal, or photomechanical effects may be used to create localized damage. An inactivation of proteins by laser induced radical formation is possible with a damage radius of a few nanometer [44], by irradiating conjugates of malachite green and antibodies. Photothermal damage is an attractive mechanism for microeffects, since the

damage range can be adjusted by changing the parameters of irradiation. Photomechanical damage, which is caused by shockwaves or explosive evaporation of water, will inevitably cause collateral damage, and is, therefore, expected to be of limited precision.

Future work will measure the threshold for evaporation at the surface of nanometer particles and compare the results with theoretical considerations based on the effect of surface tension. The denaturation of proteins in the nanosecond time range will be investigated with conjugates of proteins and absorbing micro- and nanometer sized particles. Based on data from these experiments it should be possible to predict mechanisms and parameter ranges for thermal microeffects and to investigate their potential *in vitro* and *in vivo*.

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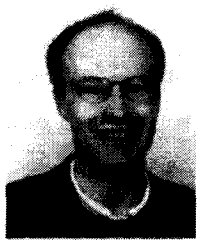

G. Hüttman was born in Herne, Germany, in 1962. He received the M.S. degree in physics in 1988, and the Ph.D. degree in physical chemistry in 1992, from the University Göttingen.

Since 1992, he is a Research Member of the Medical Laser Center in Lübeck, Germany, working in the fields of photodynamic therapy and fluorescence detection of tumors. His main research interests are now in photothermal and photochemical microeffects.

Reginald Birngruber was born in Munich, Germany. He studied electrical engineering at the Institute of Technology and physics at the Technical University, both in Munich. He received the Ph.D. degree in physics from the Johann Wolfgang Goethe University, Frankfurt, Germany.

From 1972 to 1980, he was a research member with the Department of Applied Optics, Gesellschaft für Strahlen-und Umweltforschung, Munich, a national laboratory for environmental and radiation research and from 1985 to 1992, Professor of medical biophysics with Ludwig Maximilians University, Munich. He became a visiting Professor at Harvard University, and the Division of Health Sciences and Technology, Massachusetts Institute of Technology, in 1991. As Codirector of the Medical Laser Application Program of the Wellman Laboratories of Photomedicine at Massachusetts General Hospital, he was responsible for research in the basics of ultrafast laser pulse tissue interaction, confinement of photothermal and photochemical tissue effects and laser applications in ophthalmology, as well as the cardiovascular system. Since 1992, he has been Chairman of the Medical Laser Center Lübeck and conducts research and development in the whole field of lasers in medicine and biology. He was Cochairman at the 1986 Gordon Research Conference on lasers and biology and has published more than 150 articles in laser biomedicine.