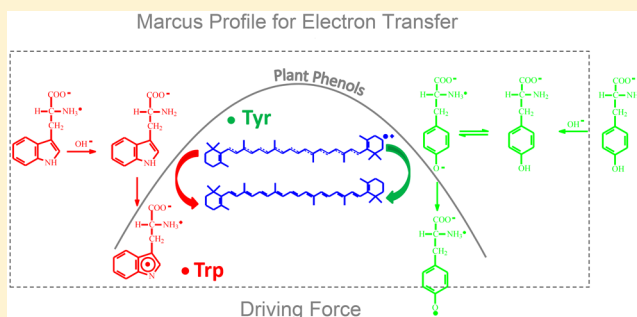


Regeneration of  $\beta$ -Carotene from the Radical Cation by Tyrosine and TryptophanHong Cheng,<sup>†</sup> Rui-Min Han,<sup>†</sup> Ming-Kuan Lyu,<sup>†</sup> Jian-Ping Zhang,<sup>\*,†</sup> and Leif H. Skibsted<sup>\*,‡</sup><sup>†</sup>Department of Chemistry, Renmin University of China, Beijing, 100872, China<sup>‡</sup>Food Chemistry, Department of Food Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

**ABSTRACT:** The phenolic amino acid tyrosine (Tyr) was found more efficient in regenerating  $\beta$ -carotene ( $\beta$ -Car) from the radical cation ( $\beta$ -Car<sup>•+</sup>) than tryptophan (Trp) in the presence of base for conditions where the reduction potentials for Trp and Tyr are comparable. Electron transfer from Tyr in 4:1 chloroform/methanol to  $\beta$ -Car<sup>•+</sup> in the presence of excess base, (CH<sub>3</sub>)<sub>4</sub>N<sup>+</sup>OH<sup>−</sup>, had a rate close to diffusion control and a second-order rate constant in agreement with the Marcus theory for electron transfer when compared to plant phenols. A maximum of 40%  $\beta$ -Car was regenerated for ten times excess of Tyr as studied by 532 nm laser flash photolysis followed by transient absorption spectroscopy in the visible and near-infrared regions. The nonregenerated fraction of  $\beta$ -Car is assigned to secondary degradation processes. For Trp, the rate constant for regeneration of  $\beta$ -Car<sup>•+</sup> was 1 order of magnitude smaller compared to Tyr and slower than expected from Marcus theory by comparison with plant phenols.

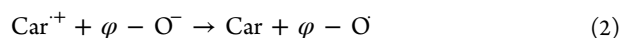


## INTRODUCTION

In plants,  $\beta$ -carotene ( $\beta$ -Car) is integrated in the photosystem II reaction center in order to protect against excessive light exposure and against oxidative stress and is for this function associated with proteins.<sup>1,2</sup> Other carotenoids like astaxanthin in shrimp and in muscles of some fish are also associated with proteins, from which astaxanthin dissociates upon heating as evidenced by the color change from gray to pink during cooking of these seafoods.<sup>3,4</sup> The function as antioxidant and radical scavenger for carotenoids in animal foods and in leafy plant foods leads to formation of carotenoid radical cations (eq 1) as a first reaction product following electron donation to oxidizing radicals.<sup>5</sup>



Besides the electron transfer (ET) mechanism, the radical adduct formation (RAF) and the hydrogen atom transfer (HAT) mechanisms with different first reaction products are crucial important for radical scavenging.<sup>6,7</sup> Carotenoid regeneration by polyphenols from the respective carotenoid radical cation (Car<sup>•+</sup>) at the water/lipid interface have been shown for an increasing number of plant phenols in their deprotonated forms (eq 2):<sup>8,9</sup>



For plant phenols, the rate of electron transfer to  $\beta$ -Car<sup>•+</sup> could be accommodated within the Marcus theory.<sup>9</sup> For the most reducing plant phenols in their phenolate form, a decrease in rate was observed for decreasing reduction potentials as a

confirmation of an inverted region for a driving force larger than the reorganization energy.<sup>10</sup>

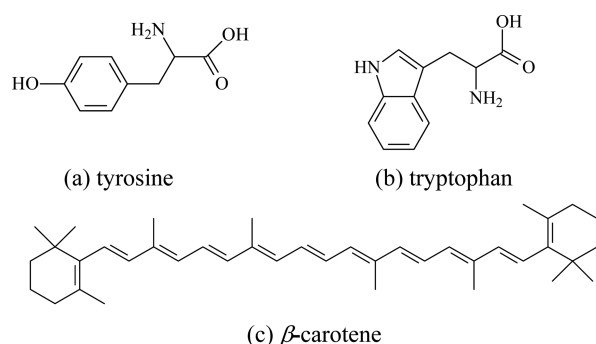
The interaction of the Car<sup>•+</sup> with reducing protein side chains remains, however, to be investigated in order to understand the role of proteins in modifying carotenoids as antioxidants and the role of proteins in protecting and regenerating carotenoids.<sup>5,11</sup> We have selected tyrosine (Tyr) and tryptophan (Trp) as possible electron donors for oxidizing radicals like Car<sup>•+</sup> for a study of the regeneration kinetics for  $\beta$ -Car as a ubiquitous carotenoid by protein amino acids using laser flash photolysis combined with transient absorption spectroscopy (Scheme 1). An understanding of carotenoid regeneration by amino acids in relation to their capability as electron donors should also help to understand the function of proteins in regeneration of carotenoids bound to proteins under oxidative stress in photosynthetic reaction center of plants and in animal-based foods during storage.<sup>1,4</sup> For physiological conditions, Tyr and Trp have comparable standard reduction potentials, still the mechanisms for electron transfer may be different due to their different structure. For Tyr, base is expected to enhance electron transfer through formation of the phenolate ion,<sup>9,12</sup> while for Trp, the possible reaction intermediate to be searched for is an indolyl radical.<sup>12,13</sup>

Received: March 19, 2015

Revised: May 7, 2015

Published: May 9, 2015

**Scheme 1. Molecular Structures of (a) Tyrosine, (b) Tryptophan, and (c)  $\beta$ -Carotene**



## MATERIALS AND METHODS

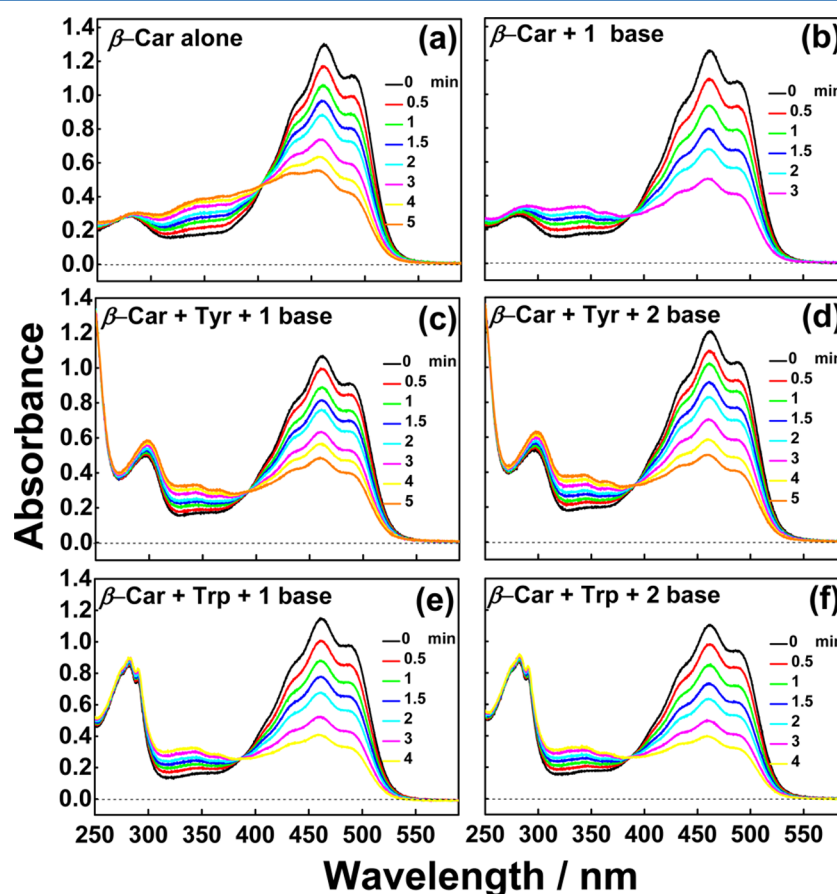
**Chemicals.** All-*trans*- $\beta$ -Car was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Tyr and Trp were purchased from Beijing OUTE Technology Co. Ltd. China. High-performance liquid chromatography (HPLC) grade methanol (J&K Scientific LTD, Beijing, China) was used as received and chloroform (Honeywell Burdick & Jackson, NJ) was used after passing through an alumina column. Deprotonated solutions of Tyr and Trp were prepared by addition 1 or 2 equiv tetramethylammonium hydroxide

((CH<sub>3</sub>)<sub>4</sub>N<sup>+</sup>OH<sup>−</sup>, 97%, Sigma-Aldrich, St. Louis, MO) to the parent neutral solutions with the amino acids dissolved in chloroform/methanol (4:1, v/v).

**Laser Flash Photolysis.** The laser flash photolysis apparatus has been described in detail elsewhere.<sup>8,9</sup> Briefly, the excitation laser pulses at 532 nm (3 mJ/pulse, 7 ns, 10 Hz) and the probe light provided by a xenon lamp (CW 300W) were used for laser flash photolysis. The optical path length of the flow cuvette ( $\sim 60$  mL) was 5 mm. The kinetics at 390–1000 nm were detected with a photodiode (model S3071, Hamamatsu Photonics, Hamamatsu, Japan) and were stored and averaged with a digital storage oscilloscope (bandwidth 600 MHz; Teledyne LeCroyWaveSurfer 64Xs, Chestnut Ridge, NY).

The concentration of  $\beta$ -Car in this study was 10  $\mu$ M. Solution samples were used immediately after preparation under dark conditions. Anaerobic conditions were achieved by bubbling the solution with high-purity nitrogen for  $\sim 30$  min. All of the measurements were carried out in a thermostated room  $23 \pm 1$  °C.

**Kinetics Analyses.** For time evolution profiles of absorbance ( $\Delta OD(t)$ ) at 990 or 490 nm, a fast exponential increase term with a pseudo-first-order rate constant  $k_1$  and a slower exponential decrease term with a pseudo-first-order rate constant  $k_2$  were used.<sup>14,15</sup>



**Figure 1.** Steady-state absorption spectra of  $\beta$ -Car (10  $\mu$ M) (a) alone, in the presence of (b) 100  $\mu$ M base, (c) 100  $\mu$ M Tyr and 100  $\mu$ M base, (d) 100  $\mu$ M Tyr and 200  $\mu$ M base, (e) 100  $\mu$ M Trp and 100  $\mu$ M base, (f) 100  $\mu$ M Trp and 200  $\mu$ M base, in chloroform/methanol (4:1 v/v) following 532 nm laser irradiation of 3 mJ/pulse of 7 ns at a repetition of 10 Hz for indicated time followed by spectrophotometric measurement using 1 cm optical cell under anaerobic condition with constant stirring. 1 or 2 base indicates 1 or 2 equiv of (CH<sub>3</sub>)<sub>4</sub>N<sup>+</sup>OH<sup>−</sup> relative to the concentration of Tyr or Trp.

$$\Delta OD(t) = A_1 \cdot \exp(-k_1 t) + A_2 \cdot \exp(-k_2 t)$$

For recovery kinetics of  $\beta$ -Car at 490 nm, the  $\Delta OD(t)$  traces were analyzed by the following expression including a fast exponential bleaching term with a pseudo-first-order rate constant  $k_3$  and a slower exponential recovery term with a pseudo-first order rate constant  $k_4$ :<sup>14,15</sup>

$$\Delta OD(t) = A_3 \cdot (1 - \exp(-k_3 t)) + A_4 \cdot \exp(-k_4 t)$$

$A_n$  indicates the respective amplitude.

## RESULTS

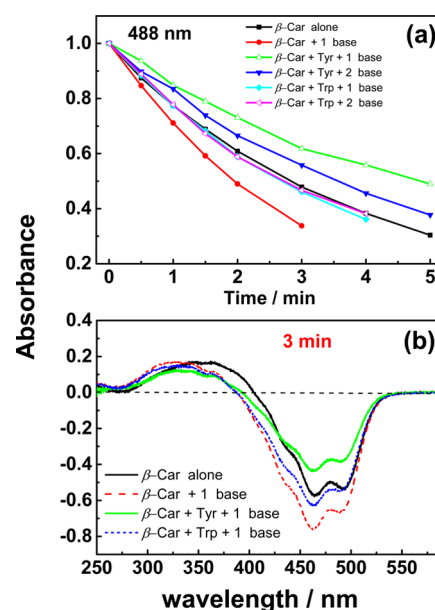
Carotenoids dissolved in electron-accepting solvents like chloroform and subsequently exposed to visible light have often been used as a convenient method for the study of  $\text{Car}^{\bullet+}$  and their further reactions using both steady-state photochemical methods and time-resolved techniques.<sup>8,9,16,17</sup> It is known that the optically allowed singlet excited state  $S_2(1B_u^+)$  of ionic character can yield  $\text{Car}^{\bullet+}$  by transferring electron to electron-accepting solvents in an ultrashort time scale ( $\sim 100$  fs).<sup>15,18</sup>

$\beta$ -Car (10  $\mu\text{M}$ ) dissolved in 4:1 (v/v) chloroform/methanol as solvent was found to bleach upon 532 nm monochromatic laser light exposure as seen in Figure 1a. The degradation of  $\beta$ -Car by light in electron-withdrawing solvents is known to proceed via a singlet excited state to yield the one-electron oxidized radical cation,  $\beta\text{-Car}^{\bullet+}$ , as seen as the reaction of eq 1.<sup>14–16</sup> Further reaction of  $\beta\text{-Car}^{\bullet+}$  results in bleaching due to disruption of the conjugated backbone of  $\beta$ -Car, as also seen from the increase of absorbance intensity at around 350 nm (Figure 1a). Addition of 100  $\mu\text{M}$  base  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  to the  $\beta$ -Car solution was found distinctly to promote  $\beta$ -Car photodegradation as seen from Figure 1b, while the presence of 100  $\mu\text{M}$  Tyr together with 1 or 2 equiv base clearly protected  $\beta$ -Car against light-induced degradation as seen from the steady-state spectra for increasing time of light exposure (Figure 1c,d). In contrast, the presence of 100  $\mu\text{M}$  Trp together with 1 or 2 equiv base had a less significant effect on the photodegradation of  $\beta$ -Car (Figure 1e,f). The enhancement of the photodegradation by the presence of base could reflect the formation of an ion pair of the carotene radical cation as a reactive intermediate for further degradation via a neutral carotene radical:<sup>17</sup>

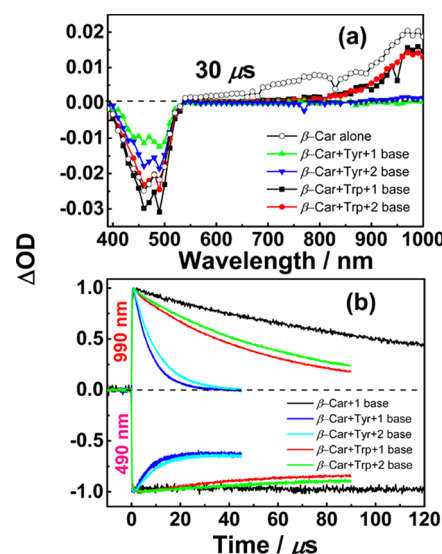


In contrast, the presence of base together with Tyr protected  $\beta$ -Car significantly for both 1:1 and 1:2 Tyr/ $\text{OH}^-$  ratios. A similar effect was seen for Trp and base, although to a lesser degree, as is seen from the time traces of absorption at 488 nm in Figure 2a,b.

Transient absorption spectra provide more clear insight into the mechanism behind the effect of base, and of Tyr and Trp in the presence of base; see Figure 3a,b. In the near-infrared region (800–1000 nm) for  $\beta$ -Car without addition of base or Tyr and Trp, the absorption recorded at 30  $\mu\text{s}$  has a characteristic maximum at 990 nm for  $\beta\text{-Car}^{\bullet+}$ , which decays in the ms time regime.<sup>14,15</sup> Transient spectra for the same delay time in the presence of Tyr and Trp with bases in a 1:1 or 1:2 ratio indicate that less  $\beta\text{-Car}^{\bullet+}$  is observed as a result of photolysis. The better protection of  $\beta$ -Car seen for Tyr compared to Trp in the presence of base was found to correlate with a larger bleaching recovery for  $\beta$ -Car at 390–550 nm in the presence of Tyr than in the presence of Trp. The decay of



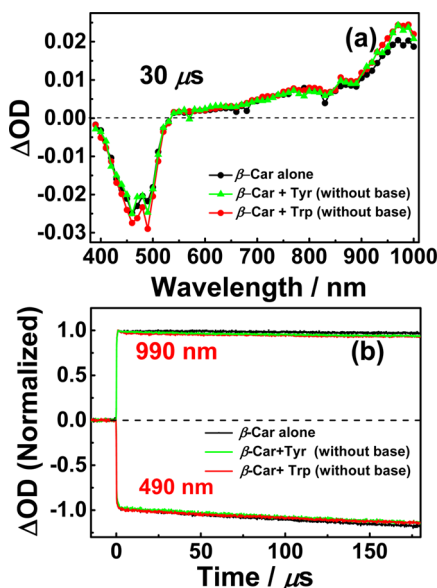
**Figure 2.** (a) Time evolution profiles for absorbance at 488 nm and (b) difference spectra between 3 and 0 min for  $\beta$ -Car in the presence of Tyr or Trp and base during 532 nm pulsed irradiation from Figure 1a,b,c,e. 1 or 2 base indicates 1 or 2 equiv  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  relative to the concentration of Tyr or Trp.



**Figure 3.** (a) Transient absorption spectra at 30  $\mu\text{s}$  and (b) normalized time evolutions of absorbance at 990 and 490 nm for  $\beta$ -Car (10  $\mu\text{M}$ ) alone, with base, and in the presence of Tyr (100  $\mu\text{M}$ ) or Trp (100  $\mu\text{M}$ ) with 1 or 2 equiv of  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  (1 or 2 base) in chloroform/methanol (4:1 v/v) binary solvent following 532 nm pulsed photoexcitation as determined under aerobic condition with constant stirring using an 0.5 cm optical cell.

the  $\beta\text{-Car}^{\bullet+}$  at 990 nm is paralleled by the recovery of the  $\beta$ -Car absorption as monitored at 490 nm, as may be seen from Figure 3b. A permanent and unrecovered bleaching excludes  $\beta\text{-Car}^{\bullet+}$  regeneration by base, although the decay of  $\beta\text{-Car}^{\bullet+}$  is accelerated to some extent by the presence of base alone, which is further in agreement with radical adduct formation in the reaction of eq 3. In contrast, the decay of  $\beta\text{-Car}^{\bullet+}$  as monitored at 990 nm and the recovery of ground state  $\beta$ -Car as monitored at 490 nm are only little affected by the presence of

Tyr or Trp without base addition (Figure 4a,b). For all conditions, a slow decay at the same rate is seen for  $\beta\text{-Car}^{\bullet+}$ . A

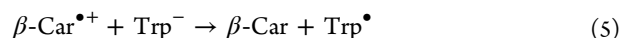
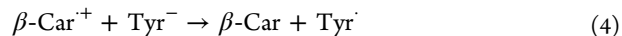


**Figure 4.** (a) Transient absorption spectra at 30  $\mu\text{s}$  and (b) normalized time evolutions at 990 and 490 nm for  $\beta\text{-Car}$  (10  $\mu\text{M}$ ) alone, with neutral Tyr (100  $\mu\text{M}$ ) or Trp (100  $\mu\text{M}$ ) in chloroform/methanol (4:1 v/v) following 532 nm pulsed photoexcitation as determined using 0.5 cm optical cell under aerobic condition with constant stirring.

secondary, slower bleaching is also noted for  $\beta\text{-Car}$  independent of the presence of Tyr or Trp in the absence of base.

For addition of base, both the decay of  $\beta\text{-Car}^{\bullet+}$  and the recovery of  $\beta\text{-Car}$  are enhanced by Trp and especially by Tyr (Figure 3b). The rate constants for the reaction of  $\beta\text{-Car}^{\bullet+}$  with Tyr and Trp in the presence of base are collected in Table 1 calculated from the kinetic traces shown in Figure 3b. Both the decay and the recovery corresponding to the rate constant  $k_2$  and  $k_4$ , respectively, are found to agree with pseudo-first-order kinetics. For  $\beta\text{-Car}^{\bullet+}$  the decay kinetics following the initial formation of the radical cation was described by use of a biexponential function for fitting, but only the decay constants are presented in Table 1 for analyzing the interaction of  $\beta\text{-Car}^{\bullet+}$  with Tyr and Trp. The  $\beta\text{-Car}^{\bullet+}$  disappears completely after  $\sim 40$   $\mu\text{s}$  in the presence of Tyr, while  $\beta\text{-Car}$  is only partly ( $\sim 40\%$ ) recovered, indicating that a competing reaction is operating for the degradation or that a secondary bleaching is contributing

probably involving solvent radicals following initial  $\beta\text{-Car}^{\bullet+}$  production.<sup>17</sup> Trp has less effect on both the rate of decay of  $\beta\text{-Car}^{\bullet+}$  and the rate of recovery of  $\beta\text{-Car}$  as compared to Tyr. Accordingly, a lower degree of recovery of  $\beta\text{-Car}$  was detected for Trp compared with Tyr for comparable time resolution. The scavenging of  $\beta\text{-Car}^{\bullet+}$  and the recovery of  $\beta\text{-Car}$  are enhanced by Tyr or Trp in the presence of base as seen from Figure 3b. The coupled radical decay and ground state recovery are accordingly assigned to the reaction of  $\beta\text{-Car}^{\bullet+}$  with the deprotonated Tyr or Trp according to



This assignment is further supported by the similar trend in the rate constants for the decay of  $\beta\text{-Car}^{\bullet+}$  and the recovery of  $\beta\text{-Car}$  as dependent on the amino acid concentration, although the significant difference in rates of decay of  $\beta\text{-Car}^{\bullet+}$  and of recovery of  $\beta\text{-Car}$  indicates that a parallel recovery reaction is operating. For all recovery reactions, and for the decay of  $\beta\text{-Car}^{\bullet+}$  in the presence of Tyr together with base, no effect of the presence of oxygen was seen. The presence of oxygen has an effect only for base assisted decay of  $\beta\text{-Car}^{\bullet+}$  through the reaction of eq 3, an effect also seen in the presence of Trp together with base with its slower reaction, but not for the faster reaction with Tyr in the presence of base; see Figure 5.

For both Tyr and Trp, the scavenging rate of the  $\beta\text{-Car}^{\bullet+}$  and the recovery of  $\beta\text{-Car}$  increased with increasing concentrations; see Figure 6a,b and Table 1. The increase of rate constants in both reactions is paralleled by an increase in recovery of  $\beta\text{-Car}$  as seen in Figure 7. The rate of decay for  $\beta\text{-Car}^{\bullet+}$  in the presence of Tyr seems to reach a limiting value for increasing concentration of Tyr. For Trp, a far higher concentration apparently is required to reach such saturation and is out of the concentration region studied. Notably for all conditions, the rate of radical decay is slower than ground state recovery.

## DISCUSSION

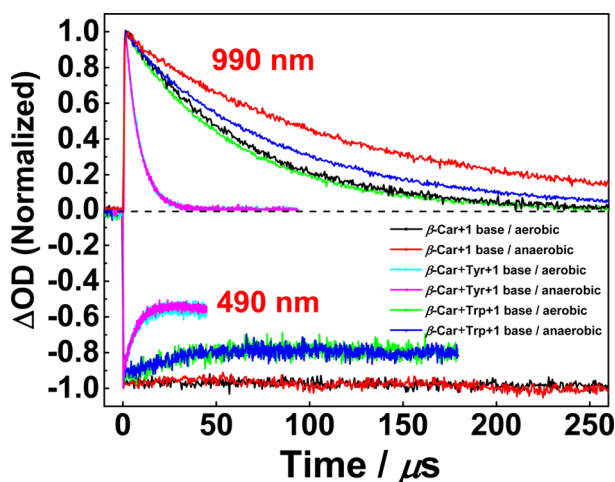
The efficiencies for Tyr and Trp regenerating  $\beta\text{-Car}$  from  $\beta\text{-Car}^{\bullet+}$  depend on the presence of base. The standard reduction potential for  $\beta\text{-Car}^{\bullet+}$  is 1.06 V versus NHE in water and independent of hydrogen ion concentration as may be concluded from the reaction of eq 1.<sup>5</sup> The standard reduction potential for the Trp radical is 1.015 V at pH = 7 in water, but decreases with increasing pH and is 0.65 V at pH = 13.<sup>13</sup> Tyr is more reducing for pH = 7 with  $E^\circ = 0.93$  V for the tyrosyl radical, but decreases only to 0.72 V at pH = 13.<sup>13</sup> Solvent

**Table 1.** Observed Pseudo-First-Order Rate Constants ( $k_{\text{obs}}/\text{s}^{-1}$ ) for Reduction of  $\beta\text{-Car}^{\bullet+}$  as Measured at 990 nm, and for Regeneration of  $\beta\text{-Car}$  as Measured at 490 nm for Excess of Tyr or Trp in the Presence of 1 or 2 equiv of  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  Relative to Tyr or Trp in (4:1 v/v) Chloroform/Methanol at 23°C

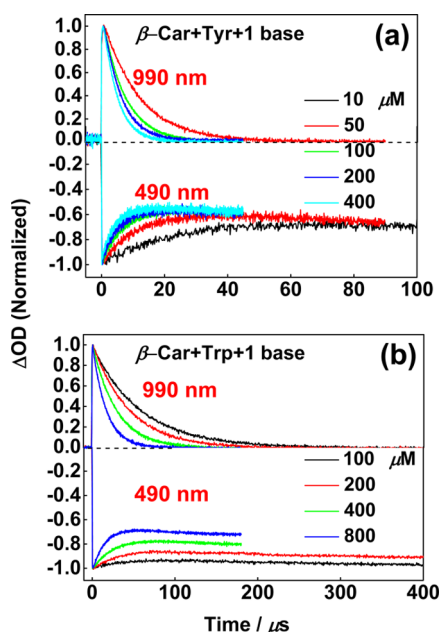
compound	$\lambda/\text{nm}$	$c_{\text{base}}$	$k_{\text{obs}}^a/\text{s}^{-1}$					
			10 $\mu\text{M}$	50 $\mu\text{M}$	100 $\mu\text{M}$	200 $\mu\text{M}$	400 $\mu\text{M}$	800 $\mu\text{M}$
Tyr	990	1 equiv	$4.3 \times 10^4$	$8.7 \times 10^4$	$1.4 \times 10^5$	$1.8 \times 10^5$	$2.2 \times 10^5$	
		2 equiv			$1.0 \times 10^5$			
Trp	490	1 equiv	$7.5 \times 10^4$	$1.3 \times 10^5$	$1.7 \times 10^5$	$2.2 \times 10^5$	$2.8 \times 10^5$	
		2 equiv			$1.6 \times 10^4$			
	990	1 equiv			$1.7 \times 10^4$	$2.2 \times 10^4$	$3.4 \times 10^4$	$6.0 \times 10^4$
		2 equiv			$1.6 \times 10^4$			
	490	1 equiv			$3.5 \times 10^4$	$3.7 \times 10^4$	$4.7 \times 10^4$	$7.4 \times 10^4$

<sup>a</sup> $k_{\text{obs}}$  represents  $k_2$  and  $k_4$  at 990 and 490 nm, respectively; see Kinetics Analyses.





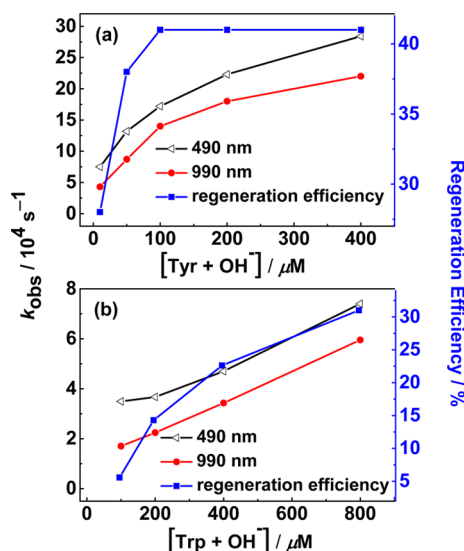
**Figure 5.** Normalized time evolutions at (a) 990 nm and (b) 490 nm for  $\beta$ -Car (10  $\mu$ M) in the presence of base (100  $\mu$ M) and in the presence of Tyr (100  $\mu$ M) or Trp (100  $\mu$ M) with 1 equiv of base relative to Tyr or to Trp following 532 nm pulsed photoexcitation as determined using 0.5 cm optical cell under aerobic or anaerobic condition both with constant stirring. One base is 1 equiv  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  relative to the concentration of Tyr or Trp.



**Figure 6.** Normalized time evolutions at 990 and 490 nm for  $\beta$ -Car (10  $\mu$ M) in the presence of different concentration (a) Tyr (10–400  $\mu$ M) with 1 equiv of base and (b) Trp (100–800  $\mu$ M) with 1 equiv of base in chloroform/methanol (4:1, v/v) solutions following 532 nm pulsed photoexcitation as determined using 0.5 cm optical cell under aerobic condition and constant stirring. 1 base is 1 equiv  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  relative to the concentration of Tyr or Trp.

effects may be important, but in the presence of base, the two amino acids become of equal reducing capacity.

For a series of plant phenols in the presence of base, the maximal regeneration of  $\beta$ -Car from  $\beta$ -Car $^{\bullet+}$  has been found to be 40%.<sup>9</sup> The regeneration was further found to depend on the driving force, as calculated from the standard reduction potentials. The optimal conditions for regeneration correspond to the reorganization energy in the donor–acceptor complex of the plant phenolate and  $\beta$ -Car according to the Marcus



**Figure 7.** Effect of concentration of (a) Tyr and (b) Trp on pseudo-first-order rate constant for regeneration of  $\beta$ -Car (10  $\mu$ M) from the radical cation as followed by radical decay at 990 nm and by bleaching recovery at 490 nm together with recovery fraction of  $\beta$ -Car (10  $\mu$ M) in the presence of 1 equiv of  $(\text{CH}_3)_4\text{N}^+\text{OH}^-$  relative to Tyr or Trp in chloroform/methanol (4:1, v/v) following 532 nm pulsed photoexcitation under aerobic condition and constant stirring.

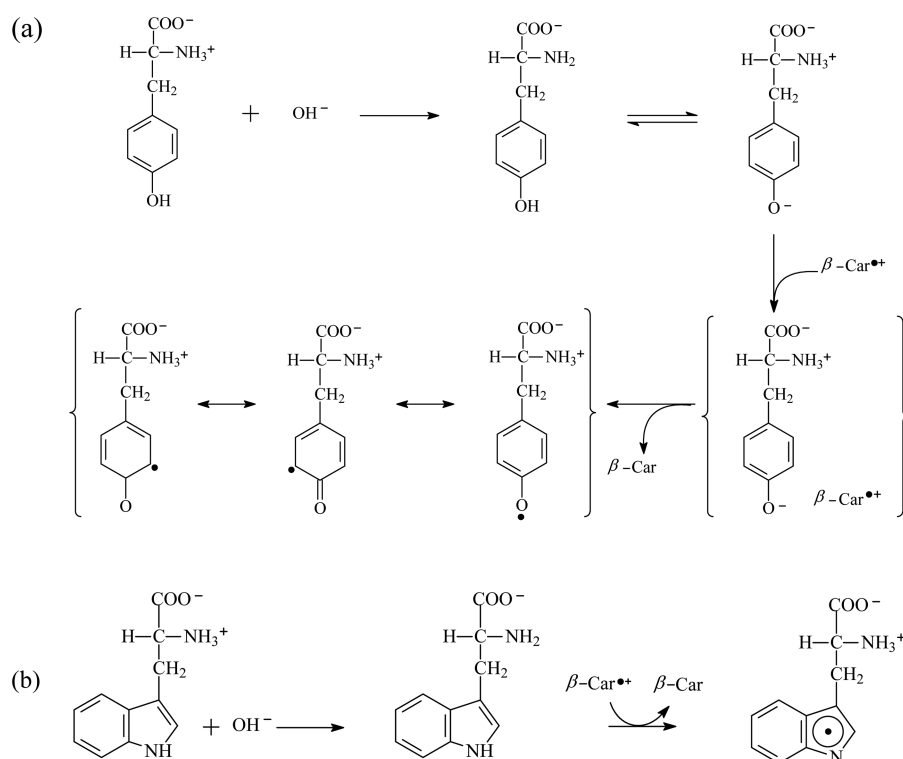
theory.<sup>8,9</sup> Syringic acid with  $E^\circ = 0.71$  V (vs NHE at pH = 7.0) thus gave the perfect match for charge distribution in the donor–acceptor transition state corresponding to a maximal rate constant with  $k_2 = 6.3 \times 10^9$  L mol $^{-1}$  s $^{-1}$  in 9:1 (v/v) chloroform/methanol close to the diffusion limit.<sup>9</sup> Both less reducing and more reducing plant phenols had slower rate for electron transfer in agreement with the Marcus theory confirming the existence of an inverted region for the most reducing phenols.<sup>10</sup> Tyr with  $E^\circ = 0.93$  V has a smaller driving force than syringic acid and was found to reduce  $\beta$ -Car $^{\bullet+}$  slightly less efficiently with  $k_2 = 1.9 \times 10^9$  L mol $^{-1}$  s $^{-1}$ . Trp with  $E^\circ = 1.02$  V at pH = 7 close to  $E^\circ = 1.06$  V, the value for  $\beta$ -Car, had a very small driving force, similar to the driving force for salicylic acid with  $E^\circ = 1.16$  V as reductant.<sup>19</sup> The resulting small driving force at pH = 7 for Trp is, however, increasing for increasing base concentration and is becoming comparable to that of Tyr, but still Trp reduces the carotenoid radical cation with a rate constant 1 order of magnitude smaller than Tyr.

For both Tyr and Trp, electron transfer to  $\beta$ -Car $^{\bullet+}$  was not observed in the absence of base in agreement with what was found in a series of plant phenols and anti-inflammatory salicylates of relevance for skin health.<sup>9</sup> For both Tyr and Trp, the reduction potential decreases for increasing pH as found for water as solvent:<sup>12,13</sup>



while the reduction potential of  $\beta$ -Car $^{\bullet+}$  is independent of pH. However, for Tyr and most of the plant phenols investigated,  $E^\circ$  is significantly lower than the  $E^\circ$  for  $\beta$ -Car $^{\bullet+}$ . Still, no electron transfer is occurring between  $\beta$ -Car $^{\bullet+}$  and the phenol as electron donor without base addition.

A precursor complex between the carotenoid radical cation and the phenolate anion is accordingly suggested to be crucial for formation of the donor–acceptor transition state for which

Scheme 2. Mechanism of  $\beta$ -Car Regeneration from Radical Cation ( $\beta$ -Car $^{\bullet+}$ ) by Excess of (a) Tyr and (b) Trp in the Presence of Base

a match of the reorganization energy ensures a maximal rate according to the Marcus theory for electron transfer.<sup>10</sup> In addition to this kinetic factor, resonance stabilization of the phenoxyl radical following electron transfer may be important as a thermodynamic factor as is evident from the decreasing value of  $E^\circ$  in the presence of base. For Trp without the phenolic group, the effect of base is less significant and it is suggested that formation of a precursor complex aided by electrostatic binding prior to the transition state for electron transfer is less facile for Trp as compared to the plant phenols and Tyr. The reduction potential of the Trp radical decreases, however, as a thermodynamic factor important for the electron transfer and comparable to the reduction potential of the Tyr radical. Trp is stabilized in its oxidized form for increasing base concentration as an indolyl radical as evidenced by the decreasing value of the reduction potential at higher pH, but with less important for kinetics as evidenced by a smaller effect on rate for electron transfer.<sup>12,13,20,21</sup> The equilibrium between the amino/phenol form of Tyr and the ammonium/phenolate form may be shifted in favor of the amino/phenol form; still, the form with the phenolate ion may be the most reactive in forming the precursor complex for electron transfer (see Scheme 2a).

The maximal regeneration of a carotenoid from the carotenoid radical cation is slightly above 40% for the most efficient of the plant phenols and reducing amino acids investigated. This apparent limit for a regeneration close to 50% may, however, be due to concomitant formation of a solvent radical in a 1:1 ratio relative to the carotenoid radical cation inducing a secondary bleaching of the carotenoid on a somewhat longer time scale.<sup>17</sup> Since the primary bleaching, which is subject to the regeneration by the reducing phenols or amino acids, accounts for only 50% of the total bleaching, this

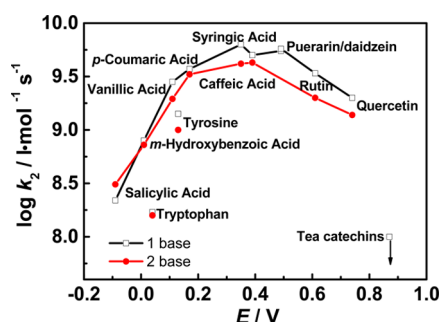
degree of regeneration represents the upper limit for the regeneration in an electron acceptor solvent.<sup>9,17</sup>

The rate constant for the regeneration of  $\beta$ -Car by both Tyr and Trp is larger than for the decay of the  $\beta$ -Car $^{\bullet+}$  for all concentration of Tyr and Trp investigated (see Figure 7 and Table 1), indicating that other reaction paths not involving the initially formed  $\beta$ -Car $^{\bullet+}$  are contributing to the initial bleaching and subsequent regeneration. One obvious reaction path would involve deactivation of an excited state of  $\beta$ -Car forming a solvent complex with absorption shifted to a shorter wavelength as a precursor to the  $\beta$ -Car radical cation.<sup>17</sup> A nonlinear dependence of the pseudo-first-order rate constants on decay of  $\beta$ -Car $^{\bullet+}$  and for recovery of  $\beta$ -Car as a function of Tyr concentration is seen in Figure 7a and to a lesser degree for Trp in Figure 7b. This could indicate formation of a precursor complex in a pre-equilibrium prior to electron transfer. A similar observation of a limiting rate for electron transfer for increasing concentration was also found for reduction of several carotenoid radical cations by isoflavonoid dianions.<sup>8</sup>

Oxygen was found to have an effect on the slower decay of  $\beta$ -Car $^{\bullet+}$  in the presence of base and in the presence of Trp and base, but not for the faster decay of  $\beta$ -Car $^{\bullet+}$  in the presence of Tyr and base. Oxygen is known not to affect the rate of decay of  $\beta$ -Car $^{\bullet+}$  in the absence of base,<sup>22</sup> but seems to promote the reaction of the neutral radical formed in the reaction of eq 3. Neutral radicals of compounds with many conjugated bonds, like carotenoids have been suggested to bind oxygen at atmospheric oxygen pressure activating oxygen, in effect becoming prooxidative.<sup>23,24</sup> Such effects could explain the increased decay of the  $\beta$ -Car $^{\bullet+}$  for the presence of oxygen. Due to the efficient competition by Tyr for reaction with  $\beta$ -Car $^{\bullet+}$ , no effect of oxygen is seen for Tyr, but for the slower reacting Trp.

Carotenoids are increasingly being recognized as important for synergistic effect with other antioxidants like plant phenols and vitamin antioxidants.<sup>25,26</sup> Proteins may be important for such interaction as carotenoid may bind to proteins and become more exposed to aqueous solutions and hydrophilic phenols. Similar positive effects for phenolic antioxidants like the tea catechins have been obtained by increasing their hydrophilicity through derivitization.<sup>27</sup> Such binding may also involve electron transfer to protein side chains and along the protein backbone. Such interaction may affect the often delicate balance of pro- and antioxidative of both carotenoids and plant phenols.<sup>25–27</sup>

If the the rate constants for electron transfer to  $\beta$ -Car<sup>•+</sup> from plant phenols, isoflavonoids, and anti-inflammatory salicylates are combined with the rate constants for electron transfer from Trp and Tyr, the picture seen as Figure 8 is obtained showing



**Figure 8.** Marcus theory plots of rate constant for electron transfer from plant phenols<sup>6,16</sup> and reducing amino acids to  $\beta$ -Car<sup>•+</sup> in the presence of base as a function of driving force expressed as difference in standard reduction potentials between  $\beta$ -Car<sup>•+</sup> and electron donor. The point for the tea catechins in the inverted region represents an upper limit for the rate constant.<sup>28</sup>

the dependence of rate constants on  $\Delta E$  as an indication of driving force.<sup>8,9,19</sup> The rate constants obtained for Tyr confirm the free energy relationship established according to the Marcus theory for the plant phenols with the further addition of rate constants for salicylic acid, puerarin, and daidzen, and an upper limit for the rate constants of the four common tea catechins.<sup>8,9,19,28</sup> In contrast, the rate constant for Trp is smaller than predicted by this free energy relationship indicative of a different reaction mechanism for this reducing amino acid lacking a phenolate for establishing an ion pair as a precursor complex. The tea catechins, i.e., (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate, and (–)-epigallocatechin gallate, are all more reducing with a standard reduction potential at pH around 7 in the range 0.19 to 0.30 V. These plant phenols do not regenerate  $\beta$ -Car from  $\beta$ -Car<sup>•+</sup> but form addition products between  $\beta$ -Car and the individual catechin in a competing reaction.<sup>28</sup> The rate of decay of  $\beta$ -Car<sup>•+</sup> has a second-order rate constants around  $10^8 \text{ L mol}^{-1} \text{ s}^{-1}$  for the four catechins. The complete lack of regeneration of  $\beta$ -Car indicates an upper limit for the rate constant for electron transfer between  $\beta$ -Car<sup>•+</sup> and catechins of this value. The catechins are clearly in the inverted region for electron transfer from a plant phenolate to  $\beta$ -Car<sup>•+</sup> as is seen for Figure 8. Other reactions are now becoming dominating such as the radical addition reaction.

## CONCLUSIONS

The phenolic amino acid Tyr was found more efficient in regenerating  $\beta$ -Car from the  $\beta$ -Car<sup>•+</sup> than Trp in the presence

of base for conditions, where the reduction potentials for Trp and Tyr are comparable and the driving forces accordingly are similar. This kinetic preference may explain why tyrosine rather than tryptophan has been selected in the protein interacting with  $\beta$ -Car in the protective mechanism in the photoreceptor against oxidative stress. The driving force will, however, depend on pH, and in proteins, the reverse reaction between a tyrosyl radical and  $\beta$ -Car may also become important in a protective mechanism as a fast electron transfer.

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work has been supported by the Natural Science Foundation of China (grant no. 21173265), the Fundamental Research Funds for the Central Universities and the Research Fund of Renmin University of China (RUC nos.10XNI007 and 14XNLQ04, 14XNH058).

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